# सायनोबैक्टेरियम एनाबीना डोलियोलम की वृद्धि एवं कार्यिकी पर क्लोराइड एवं सल्फेट प्रेरित लवणता के प्रभाव का अध्ययन

# STUDIES ON THE EFFECT OF CHLORIDE AND SULPHATE INDUCED SALINITY ON GROWTH AND PHYSIOLOGY OF THE CYANOBACTERIUM *ANABAENA DOLIOLUM*

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# STUDIES ON THE EFFECT OF CHLORIDE AND SULPHATE INDUCED SALINITY ON GROWTH AND PHYSIOLOGY OF THE CYANOBACTERIUM *ANABAENA DOLIOLUM*

By

**KRUTIKA PATIL**

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## CERTIFICATE

This is to certify that the work incorporated in the thesis entitled **"Studies on the effect of chloride and sulphate induced salinity on growth and physiology of the cyanobacterium *Anabaena doliolum*"** submitted in partial fulfillment of the requirement for the degree of **Master of Science in Microboilogy** of the **Post Graduate School, ICAR- Indian Agricultural Research Institute, New Delhi**, is a record of bonafide research carried out by **Ms. Krutika Patil (Roll No. 20989)** under my guidance and supervision and, that no part of this dissertation has been submitted by her for any other degree or diploma.

All assistance and help received during the course of this investigation has been duly acknowledged.

Place: New Delhi
Date: 02/11/19

**(Dr. Gerard Abraham)**
Chairman
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DEDICATED
TO
AMMA,
APPA AND
MY LITTLE BROTHER

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*Chapter 1*

# INTRODUCTION

The population is increasing steadily and in order to fulfill the demand of ever growing population, the crop productivity is not keeping pace with it. To make the matters worse agricultural fields are shrinking as they are now being exploited for non-agrarian purposes. Therefore, to increase productivity of the agricultural fields, one needs to either add chemical fertilizers or enhance the population of microflora. In this context, the cyanobacteria are important as they are able to fix atmospheric nitrogen and carbon and are primary producers in any ecosystem. They are also used as a source of food and thus offer great promise in the future.

The cyanobacteria are considered to be algae and they include oxygen evolving organisms with variation in the cellular organization. These organisms are one of the most ancient living entities to emerge on Globe (Sheridan *et al.,* 2003). They are also known as cyanophytes, myxophytes, cyanophycophytes etc. They have been incorporated in the category of photosynthetic bacteria because they exhibit the characters of prokaryotes and evolve oxygen during photosynthesis (Stanier and Van Niel, 1962). The cyanobacteria are also extensively distributed in a range of ecological habitats (Whitton and Potts, 2000). In addition to this they exhibit their ability to grow in habitats experiencing elevated temperature, Ultra violet irradiation, moisture stress, and many other stressors (Groniger *et al.,* 2000; Herrero and Flores, 2008). Cyanobacterial communities can also be found in several types of extreme environments (Fogg, 1982; Orcutt *et al.,* 1986; Ward *et al.,* 1989; de Chazal *et al.,* 1992).

One of the unique characteristics of the cyanobacteria is their capability to fix atmospheric nitrogen in free living and under symbiosis (Rai *et al.,* 2000). The strains fix atmospheric nitrogen with the help of distinct types of cells, the heterocysts harbouring the enzyme nitrogenase (Elhai and Wolk, 1990). These organisms are successful in niches where the availability of combined nitrogen is negligible. Rai and Sharma (2006) and Sharma *et al.,* (2010) observed that the blue green algae are agronomically outstanding as they have a lead position in the sustenance and improvement of the soil health. Applying cyanobacteria in the soil has been reported to contribute to organic content besides improving the capacity to hold adequate moisture

in the soil. It has also been observed that the extracellular polysaccharides produced are excreted in to the external environment which enhances several important properties of the soil. Certain non-heterocystous forms of cyanobacteria are also capable of $N_2$-fixation (Rippka *et al.,* 1979).

In recent times, these organisms are being exploited industrially and biotechnologically (Tan, 2010; Dixit *et al.,* 2013). However, increasing soil salinity is one of the major limiting factors for agricultural productivity of several crops globally. Non-judicious application of pesticides and chemical fertilizers coupled with faulty irrigation practices resulted in an increase in the salinity level of several agro-ecosystems. The microflora including cyanobacteria is under the threat of salinity. Microbial abundance, diversity, composition and functions are adversely affected by salinity. Salinity decreases the biomass production, primary production and nitrogen metabolism in cyanobacteria (Srivastava *et al.,* 2005, 2008; Rai *et al.,* 2014; Sen *et al.,* 2015). Impairment in the biosynthesis of pigments, carbon fixation, respiration, synthesis of proteins as well as changes in the membrane permeability has been the adverse consequences of salinity (Rai and Abraham 1993; Hasegawa *et al.,* 2000; Swapnil *et al.,* 2015). Various types of stressors induce the production of free radicals and cellular damages in cyanobacteria (Borsani *et al*., 2001; Swapnil *et al*., 2017). Osmotic stress induced by salinity exposure leads to lowering of water potential and accumulation of intracellular ions resulting in disturbed ion homoeostasis. Kochian and Lucas (1988) and Swapnil *et al.,* (2015) reported that disturbed ion homoeostasis further leads to changes in the membrane permeability affecting the uptake of essential ions resulting in nutrient deficiency. It was also observed that increased accumulation of $Na^+$ affects the uptake and translocation of $K^+$ and $Ca^{2+}$ (Hu and Schmidhalter 2005).

Several cyanobacteria show considerable adaptive ability to saline conditions (Jeanjean *et al.,* 1993; Pandhal *et al.,* 2009). Differential tolerance to saline conditions in cyanobacteria is related to structural characteristics (Stal 2007). Mainly, investigations on salinity stress are conducted with sodium chloride (NaCl) but in the natural environment, a combination of various salts such as sodium, calcium and magnesium as well as anions such as chlorides, sulphates and bicarbonates are also present in toxic forms (Grattan and Grieve 1999). In several parts of the country, the arable lands are irrigated with salt water due to paucity of good quality irrigation water (Nriagu, 1978).

However, increased industrialization and urbanization and over exploitation of natural resources have resulted in ecological imbalances. Further, the contaminants that enter the ecosystem as well as increasing soil salinity is a matter of great global concern. In natural ecosystems, organisms including cyanobacteria are constantly exposed to the threat of biotic and abiotic stresses and are forced to grow under sub-optimal conditions. Among the stresses, salinity is one of the most deleterious factors that lead to reduced growth and yield. Therefore, a better understanding of the physiological basis underlying salinity stress is important. Further, sulphate induced salinity is also increasing due to anthropogenic activities and from industries. In the respective ecological habitats, the organisms are exposed to selection pressure due to more than one stress factor. One stress factor may modify or alter the stress due to another. However, no attempts have ever been made to study the combined effect of chloride and sulphate induced salinity in cyanobacteria. Therefore, the present study entitled "Studies on the effect of chloride and sulphate induced salinity on growth and physiology of the cyanobacterium *Anabaena doliolum*" has conducted with the following objectives:

1. To assess the role of Chloride and Sulphate induced salinity on growth of the cyanobacterium *Anabaena doliolum*

**2.** To understand the response of nitrogen assimilation and antioxidant enzyme profile of the cyanobacterium *Anabaena doliolum* under salinity

*Chapter 2*

# REVIEW OF LITERATURE

Adverse impact of both abiotic and biotic stress factors is a serious threat to the ecosystem dynamics which is a great global concern. Enhanced salinity of the soil is a dangerous situation to agriculture limiting crop productivity. According to a recent report 19.5% of the irrigated agricultural lands worldwide is affected by salinity (FAO, 2016). In Asian countries which depend upon irrigation water for agriculture in several areas are now being under the grip of salinity. Further, salinity may directly and indirectly affect the growth of microflora associated with paddy fields especially the cyanobacteria. Two vital processes known since times immemorial, such as photosynthetic evolution of oxygen and biological nitrogen fixation is carried out by these unique organisms. It has been reported that on an average they can contribute to the nitrogen economy of paddy fields about 20-30 Kg N $ha^{-1}$ (Vaishampayan *et al.,* 2001) at no cost and are considered as biofertilizers in paddy fields (Kiran *et al.,* 2016). Salinity in general leads to adverse impact on plants as well as cyanobacteria. Adverse effects on the fertility of the soil due to increasing salinity have been reported by Oren (2008). Several essential microbial metabolic processes have been adversely affected due to salinity (Casamayor *et al.,* 2002). According to Rietz and Haynes (2003) salinity leads to negative impact on microbial diversity, community structure and dynamics of the ecosystem. Several cyanobacteria show considerable adaptive ability to salinity (Jeanjean *et al.,* 1993; Pandhal *et al.,* 2009; Yadav *et al.,* 2016). The following section is intended to provide a brief idea about the cyanobacteria and the studies performed regarding the adverse role of salinity stress in cyanobacteria.

The cyanobacteria are considered to be the most the primitive group of microorganisms evolved primarily from anoxygenic phototrophs and began the process of oxygenating Earth's atmosphere. It has been reported that they are the only unique photosynthetic prokaryotes to evolve $O_2$ (Hamilton *et al.,* 2014). According to Margulis (1970) the cyanobacteria which developed the ability to conduct oxygenic photosynthesis was later transmitted this ability to eukaryotes via the endosymbiosis 2.5–3.5 billion years ago. This in turn has resulted in the emergence of eukaryotic algae and chloroplasts of higher plants. There are ample evidences for this theory due to the similarities between chloroplasts of eukaryotic cells and cyanobacteria in relation to

genomic organization, photosynthetic pigments and their structures, carbon fixation and photorespiration (Raven *et al.,* 2003; Duglas *et al.,* 2003). These cyanobacteria are ecologically diverse and found in almost every terrestrial, freshwater and marine habitat, including extreme environmental conditions from oceans to fresh water to Antarctic lakes to hot springs (Fogg *et al.,* 1973). During the course of their evolution these organisms have been exposed to several natural as well as manmade stress conditions and this is one of the reasons responsible for their ecological success in several habitats. They play a significant role in nutrient cycling of important elements (De Ruyter and Fromme 2008). These organisms are also highly significant from an ecological perspective due to their main role as primary producers because of photosynthesis evolving oxygen and also are an important sink for $CO_2$. Furthermore some cyanobacteria are able to carry out nitrogen fixation making their existence valuable in the ecological niches (Scanlan *et al.,* 2009).

The name cyanobacteria is derived from Greek word 'kyanos' meaning blue colour. Cyanobacteria are a phylum under the domain Bacteria. They are also known as 'Blue-green algae' because of the colour developed as a result of the presence of the pigment phycocyanin. However, in modern context, the term algae is confined only to eukaryotes. However, in olden times, bacteria were also first classified as plants representing the class Schizomycetes, which along with the Schizophyceae (blue-green algae/Cyanobacteria) formed the phylum Schizophyta (Nageli, 1857). Subsequently, the cyanobacteria were classified as Monera under the kingdom Protista along with other algae (Haeckel, 1867). Later they have been reclassified as Prokaryotes by Chatton (1925). It was Stanier *et al.,* (1978) who supported the idea of classification of cyanobacteria according to the bacteriological code of nomenclature.

Many morphological characters were taken in to consideration to describe higher taxa (coccal v/s tracheal form, tapering, polarity, types of branching, dimensions, presence of akinetes, etc.). These traits however, have got evidently appeared and/or been vanished numerous times during the course of evolution of modern species and genera (Gugger & Hoffmann 2004, Schirrmeister *et al.,* 2011, Komárek 2013, Shih *et al.,* 2013). At present, the cyanobacteria are conventionally classified based on the morphology into five sections, denoted by the numerals I–V. The first three – Chroococcales, Pleurocapsales, and Oscillatoriales are not supported by phylogenetic studies. But, the latter two Nostocales and Stigonematales are monophyletic and

constitute a group of heterocystous cyanobacteria (Gugger and Hoffmann, 2004; Howard-Azzeh *et al.,* 2014). Table 2.1 depicts the classification of cyanobacteria based on phylogenetic analyses and ultrastructural patterns of thylakoids according to Komarek *et al.,* (2014).

The general observation is that the Cyanobacteria generally exhibit high level of adaptive abilities and tolerance to a large number of environmental stresses. During the course of their evolution the cyanobacteria have overcome acute stress conditions such as temperature variations, anoxic conditions, moisture stress, salinity, etc. Therefore, these organisms are considered as ideal candidates to decipher the basics of environmental perturbations (Murata and Wada 1995).

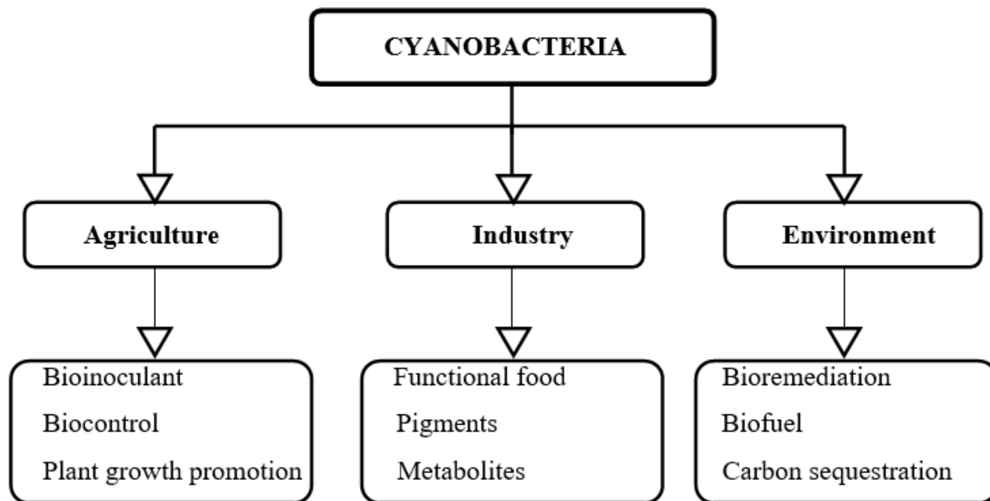
**Table 2.1: Taxonomic features on the classification of cyanobacteria (Komarek *et al.,* 2014)**

| Order | Family | Features |
|---|---|---|
| I.Gleobacteriales (no thylakoids) | Gleobacteriaceae | Polar granules, |
| II. Syneccococcales (Partial thylakoids) | Syneccococcaceae | Division in one plane |
| | Prochloraceae | Division in one plane<br>Cholorophyll b |
| | Coelospiraceae | Division in two planes<br>Polarized cells |
| | Achlarochoridaceae | Division in multiple planes<br>Chorophyll d |
| | Chamaesiphonaceae | Asymmetrical binary fission<br>Sedentary, polarized cells |
| | Pseudoanabaenaceae | Sheaths absent<br>Regular filaments |
| III.Spirulinales (Partial thylakoids) | Spirulinales | Sheaths absent<br>Coiled filaments |
| IV. Chroococcales (Irregular thylakoids) | Microcystaceae | Division in three planes<br>Aerotopes present |
| | Cyanotrichaceae | Division in one plane<br>Pseudo-filaments (sheaths) |
| | Stichosiphonaceae<br><br><br>Chroococcaceae | Asymmetrical binary fission<br>Sedentary, polarized cells,<br>exocytes present<br>Division in three or more planes<br>Colonial, aerotopes absent |

| V.Pleurocapsales (Irregular thylakoids) | Hydrococcaceae | Irregular cell division<br>Pseudo-filamentous or paranchymatous |
|---|---|---|
| | Dermocarpellaceae | Division in multiple planes<br>Baeocytes<br>polarized cells |
| | Xenococcaceae | Irregular cell division<br>Baeocytes<br>polarized cells |
| VI. Oscillatoriales (Irregular/radial thylakoids) | Oscillatoriaceae | Short cells<br>Irregular thylakoids |
| | Cyanothecaceae | Division in one plane<br>Unicellular, keritomization<br>Special cell wall structure |
| | Gomontiellaceae | Short cells<br>Special thylakoid arrangement<br>Special cell wall (cellulose) |
| | Homeotrichaceae | Polar filaments<br>False branching |
| VII. Chroococcidiopsidales (Irregular/radial thylakoid) | Chroococcidiopsidaceae | Division in three or more planes<br>Baeocytes |
| VIII. Nostocales (Irregular thylakoid) | Scytonemataceae | Isopolar filaments<br>False branching |
| | Nostocaceae | Isopolar or heteropolar filaments<br>Facultative false branching<br>Akinetes |
| | Haphalosiphanaceae | T-type true branching<br>Uni or multiseriate main filament with uniseriate branches |
| | Tolypothricaceae | Heteropolar filaments<br>False branching |
| | Capsosiraceae | Cell aggregates and filaments<br>True branching<br>Polar growth of colonies |

Some cyanobacteria have the potential capability to convert free nitrogen because of heterocysts. These cells contain nitrogenase necessary for nitrogen turn over (Singh *et al.,* 2011). Ultimately, the ammonia is excreted in to the soil in various forms

(Subramanian and Sundaram, 1986). Several non-heterocystous and unicellular cyanobacteria are also capable of fixing atmospheric nitrogen. In addition, symbiotic associated cyanobacteria are able to perform it as observed in water fern *Azolla,* cycads, *Gunnera*, etc. Therefore, the cyanobacteria are used as excellent biofertilizer for enhancing the soil fertility. The advantages of using cyanobacteria as biofertilizer is due to the very fact that they can live on very bare minimum amount of innately available inputs such as solar energy, carbon dioxide, and water (Woese, 1987; Castenholz, 2001). These biofertilizers can be very conveniently used by small scale farmers to harvest higher and sustainable yields, maintain healthier soils for future use (Higa and Wididana, 1991). They play an important role in nitrogen ecosystem. The cyanobacteria exploit sun's energy captured during the process of oxygenic photosynthesis to fix nitrogen and turn it into a form which is utilizable by the plants. Singh *et al.,* 2016) highlighted the importance of cyanobacteria as valuable resources in sustainable agriculture. Fig. 2.1 depicts the multifaceted uses of cyanobacteria in agriculture, industry and environment.




According to Issa *et al.,* (2014), the contribution of nitrogen fixed by cyanobacteria could be about 20–30 kg N $ha^{-1}$ per crop season. Further, they also contribute organic matter to the soil which is important from the perspective of a marginal farmer. *Anabaena variabilis, Nostoc muscorum, Aulosira fertissima*, and *Tolypothrix tenuis* are the most commonly observed biofertilizer strains. Increase in the N contribution to plants with cyanobacterial application has been observed (Peters *et al.,* 1977; Singh and Singh, 1987). Cyanobacterial application has been extensively

practised in China, India and Vietnam as a tool for crop yield improvement (Venkataraman, 1972; Lumpkin and Plucknett, 1982). Application of cyanobacteria improves the bioavailability of phosphorus to the plants by mobilization of insoluble organic phosphates as well as its solubilisation with the help of the phosphatase enzymes (Dorich *et al.,* 1985; Wolf *et al.,* 1985; Cameron and Julian, 1988). Plant growth promoting activity by cyanobacteria is important as they have been reported to release extracellular plant growth promoting substances (Singh and Trehan, 1973; Rodgers *et al.,* 1979; Marsalek *et al.,* 1992). It was observed that cyanobacteria such as *Anabaena, Calothrix, Cylindrospermum, Glactothece, Nostoc, Plactonema, Synechocystis,* etc. secrete auxins (Selykh and Semenova, 2000; Sergeeva *et al.,* 2002). Gibberellins production was reported in *Anabaenopsis, Cylindromum,* etc. (Mohan and Mukherji, 1978) whereas *Anabaena, Chlorogloeopsis, Calothrix,* etc. produce cytokinins (Rodgers *et al.,* 1979; Selykh and Semenova, 2000). The fast multiplication rates make them suitable for commercial application to many agriculture fields as plant growth promoters (Ruffing, 2011).

The CCUBGA, ICAR-Indian Agricultural Research Institute has developed a carrier based cyanobacterial biofertilizer comprising of four cyanobacteria such as *Anabaena, Nostoc, Aulosira* and *Tolypothrix* which is extremely popular among farmers. In addition to rice crop, cyanobacterial inoculation was found to enhance the plant shoot/root length, dry weight, and yield in wheat (Karthikeyan *et al.,* 2007, 2009). Recently Prasanna *et al.,* (2015) used cyanobacterial formulations for the improvement in Maize hybrids. Improvement in the growth and related characteristics of Chrysanthemum plants was reported with the application of consortia involving cyanobacteria (Prasanna *et al.,* 2016). All these results show that the importance of cyanobacteria as bioinoculants for crops other than rice. Another important application of cyanobacteria is as biocontrol agents due to the antagonistic effects shown by them. They have been known to produce a variety of antimicrobial compounds (Dahms *et al.,* 2006). Anti-fungal metabolites produced by the cyanobacterium *Fischerella muscicola* prevented the Brown rust due to *Uromyces appendiculatus*, powdery mildew caused by *Erysiphe graminis* and *Pyricularia oryzae* induced rice blast (Hagmann and Juttner, 1996). Antifungal effect of *Nostoc muscorum* was effective in inhibiting *in vitro* growth of cottony rot of vegetables and flowers (*Sclerotinia sclerotiorum*) (Kulik, 1995; Tassara *et al.,* 2008). Manjunath *et al.,* (2009) reported the effectiveness of *Calothrix*

*elenkenii* against damping off symptom induced by *Rhizoctonia solani*. The role of cyanobacteria in eliciting plant defence responses in maize hybrids was highlighted recently by Prasanna *et al.,* (2015).

Another important role of the cyanobacteria is their role in the reclamation of salt affected soils. Singh (1961) reported that the cyanobacteria could be applied to make the Usar soils productive. He observed that since the cyanobacteria form a thick covering surface of the soil. Further, the applied cyanobacteria help in the conservation of soil organic nutrient profile besides enhancing the water holding capacity. It is a well-established fact that the cyanobacteria improves soil permeability and aeration due to the addition of organic matter and N. Kaushik and Subhashini (1985) reported that the cyanobacteria are good candidates for the amendment of saline and alkali soils because they improve soil structure and properties by reducing the soil pH and other soil physico-chemical characteristics of the soil. Apte *et al.,* (1987) reported that the cyanobacteria are able to curtail the influx of sodium ions. The exterior arrangement of the filaments formed by the cyanobacteria on the soil binds the soil particles and at the same time entangles the soil particles at depth (Mazor *et al.,* 1996; Nisha *et al.,* 2007). The unique property of carbon and nitrogen fixation is also responsible for the enhancement of nutrient profile of the soil. According to Moisander *et al.,* (2002) *Anabaena oscillarioides, A. aphanizomenoides*, and *Microcystis aeruginosa* show considerable ability to tolerate salt ranging from 7 to 15 g/L.

Bioremediation is another important area where cyanobacteria are extensively used. Sokhoh *et al.,* (1992) observed that due to the photoautotrophic nature and capacity to fix atmospheric $N_2$ the cyanobacteria are an ideal choice for the process of bioremediation. After the process of bioremediation, the biomass may be utilized for the production of biofuel (Kumar and Singh, 2016). They have the ability to remove pesticides from various types of ecosystems (Liehr *et al.,* 1994; Al-Hasan *et al.,* 2001; Al-Hasan *et al.,* 1998, 2001). Bioremediation of heavy metals using has also been attempted employing cyanobacteria (Singh *et al.,* 2011). *Nostoc calcicola* and *Chroococcus* sp. were used for the removal of chromium by Anjana *et al.,* (2007). The observations have been extended to several other heavy metals such as copper, mercury and lead (El-Sheekh *et al.,* 2005; Cain *et al.,* 2008; Raungsomboon *et al.,* 2006).

Cyanobacteria are also ideal as candidates for bioenergy. The carbon dioxide fixed during photosynthesis is accumulated as carbonaceous lipid compounds which

are useful in the bio-fuels production. The various energy rich compounds produced by cyanobacteria are lipids, isoprenoids and carbohydrates which are used in the synthesis of biodiesel, ethanol and aviation fuel (Parmar *et al.,* 2011; Rosgaard *et al.,* 2012). Hydrogen is the fuel for future and several cyanobacteria have been exploited for the photobiological synthesis of hydrogen (Masukawa *et al.,* 2001; Parmar *et al.,* 2011; Nozzi *et al.,* 2013). Cyanobacteria such as *Anabaena, Calothrix, Oscillatoria, Cyanothece sp, Nostoc sp, Synechococcus* sp*, Microcystis* sp*, Gloeobacter* sp*, Aphanocapsa* sp*, Chroococcidiopsis* sp. and *Microcoleus* sp. The cyanobacterial biomass can be used in anaerobic digestion or fermentation to produce biogas. It was observed that the calorific usefulness of biogas is effectively boosted in the bio-methane synthesis due to the release of $CO_2$ (Hankamer *et al.,* 2007). For these operations cyanobacterial farming can be organised effectively according to the availability of space and environmental conditions to obtain high value bio-fuel products. Jansson and Northen (2010) observed that employing high temperature and $CO_2$ tolerant cyanobacterial species in can get rid of the issues related to NOx, SOx in high capacity water reservoir. Thus, the cyanobacteria offer a great solution to address the problem of climate change due to greenhouse gases released anthropogenically (Cuellar-Bermudez *et al.,* 2014). It is worth mentioning that the cyanobacterial application would be cost-effective, eco-friendly as suggested by Pandey *et al.,* (2014).

In the recent times, the cyanobacteria are used as food supplements for humans. A number of products are manufactured for the benefit of consumers. They have been used to improve the nutritional quality of a number of food commodities (Liang *et al.,* 2004). In this context, the most widely used cyanobacterial strain is *Spirulina* (*Arthrospira*) (Desmorieux and Decaen, 2005; Soletto *et al.,* 2005). It contains significant quantity of proteins (more than 60%) besides rich content of beta-carotene, thiamine, and riboflavin (Plavsic *et al.,* 2004; Prasanna *et al.,* 2010). Therefore, they are an important and prominent source of vitamin $B_{12}$ (Plavsic *et al.,* 2004; Prasanna *et al.,* 2010). Because of these reasons several countries are growing cyanobacteria for human consumption. *Spirulina* has been reported to contain a several secondary metabolites with antimicrobial activity (Kulshreshtha *et al.,* 2008). The metabolites obtained from cyanobacteria having tremendous biotechnological, industrial, pharmaceutical and cosmetic uses have been discussed in detail (Parihar *et al.,* 2017)

In spite of all these, the commercial exploitation as well as application as bioinoculant is hampered due to the increase in salinization which is a not ideal for agricultural operations. Plant growth is impaired due to salinity through water stress and toxicity due to accumulation of excess sodium and chloride ions leading to nutritional imbalance. In a recent report by FAO (2016), observed that 19.5% of the irrigated agricultural lands worldwide is affected by salinity. Soil salinity also affects the productivity of soil adversely (Wong *et al.,* 2010; Amini *et al.,* 2016). Besides, limiting agricultural crop productivity, the salinity also has adverse impacts on cyanobacteria as well. Oren (2008) reported that increasing salinity have negatively affected the fertility of the soil. Salinity has been reported to adversely affect the essential microbial metabolic processes (Casamayor *et al.,* 2002). The microbes are integral components of ecosystem and thus have a vital position in the global nutrient cycling processes involving carbon, nitrogen and sulphur (Newton *et al.,* 2011). This aspect is important in maintaining and sustaining the soil health (Lemian Liu *et al.,* 2015). It is a matter of serious concern that salinity adversely affects microbial diversity, community structure and dynamics of the ecosystem (Rietz and Haynes, 2003). Another serious problem is the increasing levels of secondary salinization caused by anthropogenic land use practises. In general, the soil salinity is due to a mixture of salts such as sodium, calcium and magnesium and anions such as chloride, sulphates and bicarbonates (Grattan and Grieve, 1999). Nriagu (1978) observed that in agricultural soils irrigated with salt water $Na_2SO_4$ is predominant. Increase in the sulphate content of the soils from various sources have also been highlighted by several workers (Freedman and Hutchinson 1980; Chang *et al.,* 1983). However, due to the dominating effect of NaCl, often the effects due to sulphate are side lined. Therefore, there is a need to investigate as to how these two salts in combination affect the cyanobacteria.

In view of these selection pressures maintaining the global food security will be a herculean task if the salinity tolerance of agriculturally important microorganisms is not understood properly. It is in this context, understanding the salinity tolerance potential of cyanobacteria is important although several cyanobacteria show considerable adaptive ability (Jeanjean *et al.,* 1993; Pandhal *et al.,* 2009). Tolerance to salt is clearly a trait involving several adaptations involving modulation of the physiology and alterations at molecular level. The cyanobacteria resort to various

mechanisms to cope with the changes in the saline environment. Morphological and genome diversity observed in cyanobacteria leads to differential tolerance to salinity (Stal, 2007). In cyanobacteria exposed to salinity photosynthetic activities such as PSI, PSII and electron transport activities were found to be inhibited (Rai *et al.,* 2014). Yadav *et al.,* (2016) also observed reduction of proteins and ion metabolism in *Anabaena* sp. under salinity. Another important process found to be sensitive to salinity is nitrogen fixation. Srivastava *et al.,* (2008) studied the adverse role of salinity with respect to the nitrogen fixing system of the cyanobacterium *Anabaena doliolum*. Studies conducted by Namsaraev *et al.,* (2018) reported the inhibition in the nitrogenase activity of both heterocystous and non-heterocystous cyanobacteria due to sodicity.

Ion homeostasis, synthesis of osmotica (sucrose, trehalose, glucosyl glycerol, glycine betaine) is important in maintaining the osmotic potential of the cells and activation of antioxidant enzymes to scavenge the free radicals (Mackay *et al.,* 1984; Hagemann, 2011). Therefore, the osmotic stress tolerance involves a number of mechanisms to maintain the ion homeostasis (Rajendran *et al.,* 2009; Isayenkov, 2012; Roy *et al.,* 2014). Accumulation of excess $Na^+$ is responsible for reduction in growth due to salinity (Hong *et al.,* 2009). Further, the increase in the cellular sodium content blocks the translocation of potassium as well as calcium (Hu and Schmidhalter 2005). Maathuis and Amtmann (1999) observed that the potassium deficiency is due to the structural similarity between $Na^+$ and $K^+$ and $Na^+$ replacing $K^+$ ions. The toxicity due to sodium ions is further reflected by the disruption of $K^+$ homeostasis leading to disruption in the activity of a number of enzymes (Zhu, 2007; Kronzucker and Britto, 2011). Potassium ions are required as an activator or a cofactor in several biochemical processes (Pervez and Ashraf 2001; Romheld and Kirkby 2010). Genc *et al.,* (2007) worked out a correlation between salt tolerance and sensitivity by estimating the amount of sodium in the tissues. The ratio of $K^+/Na^+$ is important in salt tolerance under salinity stress conditions (Munns and Tester 2008). Intracellular concentration of calcium was also found to increase under stress conditions (Kader *et al.,* 2007; Kader and Lindberg 2010). Calcium is an important cation required for membrane stability and ion channel activity (Martinez and Lauchli 1993). Swapnil *et al.,* (2015) observed enhanced salinity tolerance in the cyanobacteria is associated with proper maintenance of $Ca^{2+}/Na^+$ ratio. Therefore, it has been observed that the intracellular ions disturb the ion homoeostasis resulting in changes in the membrane permeability, uptake of

essential ions ultimately leading to nutrient deficiency (Kochian and Lucas 1988; Swapnil *et al.,* 2015). Further, differential levels of toxicity were observed due to sodium, chloride and sodium chloride as reported by Dang *et al.,* (2006). All these disorders ultimately result in disturbances in the metabolism of cyanobacteria severely (Hasegawa *et al,.* 2000; Swapnil *et al.,* 2015; Yadav *et al.,* 2015; Reich *et al.,* 2017).

Another important aspect of the salinity induced stress conditions is the generation free radicals resulting in cellular injury (Borsani *et al.,* 2001; Swapnil *et al.,* 2017). ROS such as hydrogen peroxide ($H_2O_2$), superoxide radicle ($O^-{}_2$), singlet oxygen ($^1O_2$) and hydroxyl radicle ($OH^{\cdot}$) are formed due to univalent reduction of oxygen, leads to leakage of electrons to $O_2$ (Scandalios, 2002). Apel and Hirt (2004) reported that hyper accumulation of these ROS leads to damage of cellular structure and functions ultimately resulting in cell death. However, the cells up regulate activity of defence mechanisms to counter the harmful effects of ROS. Various enzymatic antioxidant enzymes such as super oxide dismutase, ascorbate peroxidase, catalase, glutathione reductase and non-enzymatic antioxidants such as proline, ascorbate and glutathione are involved in countering the ROS induced by oxidative stress conditions (Mittler *et al.,* 2004; Sharmila *et al.,* 2017). Amor *et al.,* (2006) and Bose *et al.,* (2014) observed that salt tolerance is correlated with higher levels of anti-oxidant enzymes. The antioxidant machinery of the organism is therefore, critical in determining the response to abiotic stress tolerance.

Therefore, a critical perusal of the literature showed that scanty information is present on the biochemical response of cyanobacteria to the exposure of sulphate. Moreover, the sulphate induced toxicity has not been studied from a physiological perspective. Sulphur is an important nutrient and its assimilation in to cysteine, which is a precursor of a variety of organic compounds (Hell *et al.,* 2002; Saito 2004; Hawkesford and De Kok 2006). Moss (1978); Nriagu (1978); Chang *et al.,* (1983) and Reich *et al.,* (2017) observed that excess of sulphate salts may also be present in soils and anthropogenic as well as industrial sources can lead to excess deposition of sulphate in the soil. Paek *et al.,* (1988) observed that the toxicity induced by sulphate was severe as compared chloride. Detrimental effects of sulphate were also observed by Dixon (2007) in plants grown in environments receiving excess of sulphate in the soil. Ryrie and Jagendorf (1971) observed inhibition in photophosphorylation resulted in toxic effects on plants. Cerovic *et al.,* (1982) and Renneberg (1984) observed that the

intermediates of the sulphur reduction pathway are toxic to plant system. In a study conducted by Reich *et al.,* (2015) the biomass production in Chinese cabbage was found to affect due to sulphate toxicity. In a recent study conducted by Reich *et al.,* (2017) on *Brassica rapa*, it was observed that besides reduced biomass production, the accumulation of calcium, manganese and phosphorous were decreased due to sulphate induced salinity. Sulphate is also important in the metabolism of cyanobacteria and it was observed that supplementation of sulphur in the nutrient medium enhanced the GSH (γ-glutamyl-cystynyl-glycine) content (Schafer and Buettner 2001; Masip *et al.,* 2006). Enhancement in the synthesis of GSH moderates salinity tolerance as observed by Fatma *et al.,* (2014). The GSH acts as a powerful reducing compound and along with other antioxidative compounds impart a stable membrane structure by reducing the disulphide bonds of proteins (Johnson *et al.,* 2006; Foyer *et al.,* 2009). Ready availability of sulphate is important in its incorporation in to proteins and other important antioxidants (Foyer *et al.,* 2009; Nagahara and Wrobel 2017). Therefore, deprivation of sulphur in cyanobacteria reduces the GSH pool significantly (Cameron and Pakrasi 2010). On the other hand, its supplementation has resulted in better levels of glutathione (Fatma *et al.,* 2014). Therefore, to manage the redox pool of thiol, the sulphur nutrition is important. Under stress conditions maintenance of redox pool will be important in the modulation of stress tolerance response. Swapnil and Rai (2018) studied the physiological responses in the cynaobacterium *Anabaena fertilissima* in response to salinity induced by chloride and sulphate. They have observed that both chloride and sulphate exerted their toxic effects on the cynaobacterium. However, their investigations showed that the toxicity induced by sulphate was less toxic as compared to chloride. Studies conducted by Rai and Swapnil (2018) showed the expression of several proteins were induced by sulphate treatment as compare to chloride. They have observed that availability of sulphate during the stress conditions have resulted in increased accumulation of proteins. Therefore, the available information in the subject matter showed that despite toxicity related issues the availability of sulphate provided protection to the cells. However, it remains still uncertain and inconclusive whether NaCl and $Na_2SO_4$ alone or in combination lead to differential levels of toxicity in cyanobacteria. Such a study in cyanobacteria is pertinent because these organisms have those unique properties that make them biofertilizer and as "ecosystem engineers" (Jones *et al.,* 1994). The various factors such as salinity however, hamper the potential and restrict their exploitation in sustainable agriculture. Saline soils mostly have a

mixture of different types of salts and NaCl is the dominant salt. Because of this reason most of the studies are performed using NaCl. Therefore, the present study is important as it addresses the effect of individual as well as combined doses of NaCl and $Na_2SO_4$ in the nitrogen fixing cyanobacterium *Anabaena doliolum*.

*Chapter 3*

# MATERIALS AND METHODS

## 3.1 Experimental organism and growth conditions

*Anabaena doliolum*, was maintained at CCUBGA, ICAR-Indian Agricultural Research Institute, New Delhi, India was used for the present study. This organism has the capability to fix nitrogen and therefore the cultures were maintained in BG-11 medium and the pH of the medium was maintained at 7.5. A light intensity of 72 µmol $m^{-2}$ $s^{-1}$ and temperature of 28 $\pm$ 2 $^{0}$C was maintained for 16/8 h light and dark period.

## 3.2 Culture methods

Culture vessels for the research work were thoroughly cleaned by keeping them in acid mixture containing chromic and sulphuric acid for 24 h. After this, the glass wares were washed with water. Subsequently detergent washing was done with normal water and the culture vessels were finally cleaned using fine quality water and oven dried.

## 3.3 Chemicals

Analytical grade chemicals for the study were procured from Hi-media, Siskin Research Laborataries and Sigma Chemicals, respectively.

## 3.4 Sterilization

The culture vessels and the growth medium was sterilized by moisture under pressure (15 lb/inch$^2$) for 20 minutes in an autoclave at 121$^{0}$C.

## 3.5 Growth medium

*Anabaena doliolum* was cultured regularly in BG 11 basal medium (Stanier *et al.,* 1971). The composition of the growth medium is given in Table 3.1

**Table 3.1: Composition of BG 11 basal medium**

| Constituents | Concentration (g/L) |
|---|---|
| Sodium nitrate | 1.50 |
| Di potassium hydrogen phosphate | 0.04 |
| Magnesium sulphate | 0.075 |
| Calcium chloride | 0.036 |
| Citric acid | 0.006 |
| Ferric ammonium citrate | 0.006 |
| EDTA (di sodium magnesium salt) | 0.001 |
| Sodium carbonate | 0.02 |
| Micronutrient solution (A5 solution) | 1 ml |
| pH | 7.1- 7.3 |

**Table 3.2: Composition of A5 solution**

| Constituents | Concentration (g/L) |
|---|---|
| Boric acid | 2.860 |
| Manganese chloride | 1.810 |
| Zinc sulphate | 0.220 |
| Sodium molybdate | 0.039 |
| Copper sulphate | 0.079 |
| Cobalt nitrate | 0.0494 |

### 3.6 Salinity treatment

The experimental organism *Anabaena doliolum* was grown in BG 11 medium provided with various levels of sodium chloride (50, 100, 150, 200 and 250 mM) and sodium sulphate (50, 100, 150, 200 and 250 mM) to determine lethal concentration value ($LC_{50}$). Based on this, treatments were decided and BG 11 basal medium was supplemented with sodium chloride (150 mM) and sodium sulphate (100 mM) either individually or in combination. A control was also maintained without salinity treatment.

### 3.7 Dry weight

For the determination of dry weight, cyanobacterial culture (10 ml), after adequate stirring of the culture, was filtered using filter paper (Whatman No. 42). The collected suspension was then kept for drying in an oven ($60^o$ C) and kept for cooling to attain constant weight. Dry weight was calculated on the basis of differences in the weights (initial and final) according to Sorokin (1973).

### 3.8 Pigment profile

#### 3.8.1 Chlorophyll

The chlorophyll was estimated by the method of McKinney (1941).

**Reagent:** Acetone (90%)

**(i) Procedure**

Cultures (5 ml) were subjected to centrifugation at 3000 rpm for 10 min. After adding acetone (5 ml, 90%) to the pellet, the tubes were kept for incubation at $4^oC$ for 12 hours. After centrifugation, the optical density was measured at 650 nm and 665 nm using spectrophotometer. The following formula was used to calculate amount of chlorophyll in the cultures.

$$\text{Chlorophyll } (\mu g/ml) = (20.2 \times A_{650}) + (8.02 \times A_{665})/1000$$

$A_{650}$ and $A_{665}$ are the absorbance value at 650 and 665 nm, respectively.

### 3.8.2 Carotenoids

The carotenoids of the cyanobacterium *A. doliolum* was measured by the protocol of McKinney (1941).

**(ii) Procedure**

Cultures (5 ml) were collected by centrifugation at 3000 rpm for 10 min. After adding acetone (5 ml, 90%) the pellet was kept for incubation at $4^oC$ for 12 hours. The amount of carotenoid was calculated from the absorbance values obtained at 480 and 510 nm.

$$\text{Carotenoids } (\mu g/ ml) = 7.6 \times A_{480} - 1.49 \times A_{510}/1000$$

$A_{480}$ and $A_{510}$ the absorbance values at 480 and 510 nm, respectively.

### 3.8.3 Phycobiliproteins

The protocol according to Bennett and Bogorad (1973) was used to calculate the phycocyanin content of the cyanobacteria.

**Reagent:** Phosphate buffer (0.05 M, pH 7.5)

**(iii) Procedure**

5 ml of phosphate buffer (0.05 M, pH 7.5) was added to the pellet left after the extraction for chlorophyll and carotenoid,. Repeated freezing and thawing of the pellet was done to make the pellet colorless. Optical density was measured at 562, 615 and 652 nm respectively. 0.05 M phosphate buffer was used as blank. Quantity of phycobiliproteins was estimated. The phycobilin pigments such as phycocyanin, allophycocyanin and phycoerythrin was expressed as µg/ml of suspension.

$$\text{Phycocyanin } (\mu g/ml) = \frac{A_{615} - 0.474 \times A_{652}}{5.34}$$

$$\text{Allophycocyanin } (\mu g/ml) = \frac{A_{652} - 0.208 \times A_{615}}{5.06}$$

$$\text{Phycoerythrin } (\mu g/ml) = \frac{A_{562} - [2.41(PC) - 0.649(APC)]}{9.62}$$

Where, $A_{562}$, $A_{615}$ and $A_{652}$ are absorbance at 562, 615 and 652nm respectively.

PC = Phycocyanin, APC = Allophycocyanin

### 3.9 Estimation of filament length

Average filament length was estimated according to the procedure given by Mishra *et al.,* (2003). A drop of the cyanobacterial suspension was pipetted and placed on clean slide. Using a microscope, the filaments were viewed and the filaments (number) present in a field were recorded. To calculate the average length of filament, the cell number was counted were added up and divided by the total number.

### 3.10 Estimation of heterocyst frequency

Heterocyst frequency was also calculated (Mishra *et al.,* 2003) by measuring the number of heterocyst present per hundred vegetative cells.

$$\text{Heterocyst frequency} = \frac{\text{Total number of heterocyst}}{\text{Total number of vegetative cells}} \times 100$$

### 3.11 Estimation of intracellular $Na^+$, $K^+$ and $Ca^{2+}$contents

Intracellular sodium, potassium and calcium content estimated according to the method of Association of Official Analytical Chemists (1984). Ion contents were measured using 5 ml cultures of the cyanobacteria. The cyanobacterial cell suspension was subjected to centrifugation and the pellets collected were rinsed using sterile double distilled water. The washed cells were kept for drying and subsequently digested in 1 ml of acid mixture ($HNO_3$:$HClO_4$ mixture, 10:1, v/v) in water bath (100 $^0$C) for half an hour. Digested cultures were brought to room temperature and diluted to 5 ml with double distilled water. Debris was removed from the suspension after centrifugation and tested in a flame photometer (Systronics, 128). The final amount of sodium, potassium and calcium are expressed as μmol mg protein$^{-1}$.

### 3.12 Estimation of Protein

Protein content of the cyanobacterial cells was estimated according to the method developed by Lowry *et al.,* (1951).

**Reagents**

I. Sodium hydroxide (1 N)

II. (i) Sodium carbonate solution (5%)

(ii) Copper sulphate 0.5% in 1% of Sodium potassium tartarate.

- 2ml (ii) was mixed with 48ml of (i) prepared just before use.

III. Folin-ciocalteau reagent: The commercial reagent was diluted with an equal volume of water just before use.

IV. Standard protein: Bovine serum albumin solution was prepared in the range of 10-160 $\mu g\ ml^{-1}$.

**Procedure**

In the cyanobacterial cells (0.5 ml), 0.5 ml of 1N NaOH was added and the samples were kept in water bath (100 °C) for 5 min. Reagent II (2.5 ml) was then added and allowed to stand for 10 min at room temperature. 0.5 ml of reagent III was added and mixed. After 15 min incubation, the optical density was read at 650 nm. A standard curve was made using bovine serum albumin.

**3.13 Total Carbohydrate content**

The total carbohydrate content was quantified by the method of Spiro (1966).

**Reagents**

(i) Standard glucose solution 10-100 $\mu g\ ml^{-1}$

(ii) Anthrone reagent: Anthrone (0.1 g) + thiourea (1 g) in 100 ml of 75 % $H_2SO_4$ and kept in water bath at $85^0$ C for 20 minutes.

**Procedure**

To the cultures (1 ml), was added Anthrone reagent (4 ml) followed by incubation ($100^0$C for 15 minutes). After the requisite incubation period, the samples were cooled in tap water. Optical density was measured at 620 nm and a standard curve was prepared using glucose.

**3.14 Assay of N- assimilation enzymes**

**3.14.1 Nitrate reductase**

In the nitrogen assimilation pathway, nitrate reductase is the first enzyme catalyzing the reduction of nitrate. Nitrate reductase (NR) assay was assayed following the protocol given by Herrero *et al.,* (1981).

**Reagents**

(i) Sodium nitrate ($NaNO_3$)

(ii) Sulphanilamide (1g in 100 ml of 1:4 HCl and water)

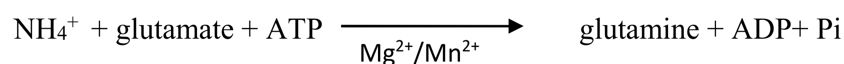
(iii) α – (N-1)) napthyl ethylene diaminedihydrochloride (NEDD), (0.2% w/v)

**Enzyme assay**

A known volume of culture was centrifuged and rinsed with sterile water and transferred in medium containing $NaNO_3$ (10 mM, pH 7.0). Cyanobacterial cell suspension (1 ml) was collected and 2 ml of sulphanilamide was added. Contents were vortexed. 2 ml α – (N-1) napthyl ethylene diaminedihydrochloride (NEDD) was added subsequently and the samples were incubated at room temperature for 30 minutes. A pink colour was developed and the absorbance was measured at 540 nm. The enzyme activity was expressed as as μ mol $NO_2$ $mg^{-1}$ of protein. A standard curve of nitrite was prepared according to Snell and Snell (1949).

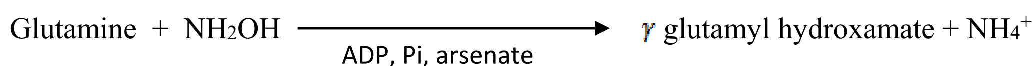
**3.14.2 Glutamine Synthetase activity**

Ammonia is converted in to glutamate with the involvement of ATP is catalyzed by cyanobacterial glutamine synthetase (L-glutamate: ammonia lyase, ADP- forming EC 6.3.1.2):



$$NH_4^+ + \text{glutamate} + \text{ATP} \xrightarrow[Mg^{2+}/Mn^{2+}]{} \text{glutamine} + \text{ADP} + \text{Pi}$$

Cyanobacterial glutamine synthetase also catalyzes the formation of γ-glutamyl hydroxamate.



$$\text{Glutamine} + NH_2OH \xrightarrow[\text{ADP, Pi, arsenate}]{} \gamma \text{ glutamyl hydroxamate} + NH_4^+$$

The qunatity of γ-glutamyl hydroxamate formed through the transferase activity was determined by the method of Shapiro and Stadtman (1979).

**Reagents**

(a) Glutamine : 0.1 M, pH 7.0

(b) Sodium arsenate : 0.1M, pH 7.0

(c) Sodium ADP : 0.01M, pH 7.0

(d) $MnCl_2$ : 0.1 M

(e) Hydroxylamine – HCl : 2.0 M

(f) Imidazole HCl buffer : 100 mM, pH 7.0

(g) γ-glutamyl hydroxamate : 10 mM standard

(h) Stop mixture: Ferric chloride (10% w/v), Trichloroacetic acid (24% w/v), 6N HCl and double distilled water in the ratio of 8:2:1:13

Reaction mixture was prepared by mixing the following volumes of the above solutions.

| | | | |
|---|---|---|---|
| I. | Imidazole buffer | : | 2.0 ml |
| II. | Glutamine | : | 1.5 ml |
| III. | $MnCl_2$ | : | 1.5 ml |
| IV. | Sodium ADP | : | 2.0 ml |
| V. | Sodium arsenate | : | 1.0 ml |
| VI. | Hydroxylamine- HCl | : | 1.5 ml |
| VII. | Water | : | 2.0 ml |

Hydroxylamine hydrochloride was neutralized with 15 ml of 2N NaOH before adding other components of the mixture.

**Enzyme assay**

Culture (0.5 ml) was mixed with 0.25 ml toluene and kept at $4^0$C and the cultures were centrifuged at 3000 rpm for 10 minutes. The toluene was completely taken out using a micropipette and suspended in imidazole buffer (0.5 ml). The assay was initiated by the addition of 0.5 ml of reaction mixture and the samples were kept at room temperature for half an hour. Reaction was completed by the adding 3.0 ml of stop mixture and absorbance was measured at 540 nm. The glutamine synthetase transferase activity was expressed as μ mol γ-glutamyl hydroxamate formed mg protein$^{-1}$

**3.14.3 Estimation of Nitrogenase Activity**

The atmospheric free nitrogen is converted to ammonia by the nitrogenase complex (EC 1.18.2.1). This reaction requires reduced ferredoxin and is obligatorily coupled to reduction of protons resulting in formation of molecular hydrogen:

$$N_2 + 8\ Fd_{red} + 8\ H^+ + 16\ ATP \rightarrow 2\ NH_3 + H_2 + 8\ Fd_{ox} + 16\ ADP + 16\ Pi$$

Nitrogenase enzyme is able to reduce several compounds having a triple bond and therefore, acetylene ($C_2H_2$) is reduced to ethylene ($C_2H_4$). Acetylene reduction assay of Stewart *et al.,* (1968) was used for the estimation of nitrogenase. The experiment was conducted in triplicate in test tubes (10 ml capacity). A known amount of culture was collected in each tubes and the acetylene concentration was maintained at 10%. The tubes were kept at $28\pm1^0$C at 2500 lux intensity with occasional stirring for 90 minutes. The reaction was completed by injecting 0.8 ml of 15% (w/v) trichloroacetic acid (TCA). Ethylene produced in the tubes was analyzed using gas chromatograph (Nucon model GLC 5500) fitted with a poropak R column and a hydrogen flame ionization detector. The nitrogenase is represented as n mol $C_2H_4$ formed mg chlorophyll$^{-1}$ h$^{-1}$.

### 3.15 Estimation of lipid peroxidation

The lipid peroxidation was estimated by measuring the total thiobarbituric acid reactive substances (TBARS) as equivalent of malondialdehyde (MDA) as suggested by Cakmak and Horst (1991)

**Reagents**

1. Trichloroacetic acid (20% w/v)
2. Thiobarbituric acid (1% w/v)

**Procedure**

Culture (10 ml) was homogenized and centrifuged at 3000 rpm for 10 min. Pellet was collected and incubated with 2 ml of TCA (20% w/v) containing TBA (1% w/v) for 30 min at 95 ºC in water bath. The reaction was stopped by cooling on ice for 10 min and the product was centrifuged at 10,000 rpm for 15 min. The absorbance of the reaction product was measured at 532nm. The amount of Mavelondialdehyde (MDA) was computed using extinction co-efficient of 155 mM$^{-1}$ cm$^{-1}$ and lipid peroxidation was expressed as n mol MDA mg$^{-1}$ protein.

### 3.16 Assay of antioxidant enzymes (enzymatic and non-enzymatic)

**Preparation of samples**

Cyanobacterial cultures were suspended in buffer (pH 7.0) containing EDTA (1mM) and polyvinyl pyrrolidone (10%). The culture samples were sonicated and centrifuged at 15,000 rpm for 30 min at 4º C. The resultant supernatant was used for the assay of the antioxidant enzyme activities.

### 3.16.1 Superoxide dismutase (EC 1.15.1.1)

The superoxide dismutase activity (SOD) was estimated according to the procedure given by Giannopolitus and Ries (1977). Reduction in absorbance of formazone made by superoxide radical and nitro-blue tetrazolium dye by the enzyme superoxide dismutase was used as the criteria to estimate superoxide dismutase activity (SOD).

**Reagents**

1. Methionine (200 mM): L-Methionine 0.298 g was dissolved in water and the volume was made up to 10 ml with double distilled water.
2. Nitro-blue tetrazolium chloride (NBT) (2.25 mM): NBT 0.0184 g was dissolved in water and the volume was made up to 10 ml with double distilled water.
3. EDTA (3.0 mM): EDTA 0.0558 g was dissolved in water and the volume was made up to 50 ml with double distilled water.
4. Riboflavin (60 μM): Riboflavin 0.0023 g was dissolved in water and the volume was made up to 100 ml with double distilled water.
5. Sodium carbonate (1.5 M): Sodium carbonate 7.95 g was dissolved in double distilled water and the volume was made up to 50 ml with double distilled water.
6. Phosphate buffer (100 mM, pH 7.8):

   Sol A: Potassium dihydrogen phosphate 6.8 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

   Sol B: Di-potassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

   Buffer was prepared by mixing 8.5 ml of sol. A and 91.5 ml of sol. B and final pH was adjusted with the help of pH meter.

**Enzyme assay**

The reaction mixture (3 ml) consisted of 13.33 mM methionine (0.2 ml of 200 mM), 75 μM nitro- blue tetrazolium chloride (NBT) (0.1 ml of 2.25 mM), 0.1 mM EDTA (0.1 ml of 3 mM), 50 mM phosphate buffer (pH 7.8) (1.5 ml of 100 mM), 50 mM sodium carbonate (0.1 ml of 1.5 M), 0.05 to 0.1 ml enzyme, 0.9 to 0.95 ml of water (final volume of 3.0 ml). Reaction was initiated by adding riboflavin (2 mM, 0.1 ml)

and keeping the tubes under fluorescent lamps (15 W) for 15 min. A colour was employed with a complete reaction mixture minus enzyme, which showed the maximum colour. The reaction was completed by turning off the light exposing the tubes in dark conditions. A complete reaction mixture (not exposed to light) served as a blank. The optical density was measured at 560 nm. The amount of enzyme able to bring down the optical density by half in comparison to the control was taken as One unit of enzyme activity (U mg $protein^{-1}$).

### 3.16.2 Ascorbate peroxidase (EC 1.11.1.11)

Ascorbate peroxidase (APX) was assayed by recording the decrease in absorbance due to ascorbic acid at 290 nm (Nakano and Asada, 1981).

**Reagents**

1. Ascorbic acid (3.0 mM): Ascorbic acid (0.0265 g) was dissolved in water and the volume was made up to 50 ml with double distilled water.
2. EDTA (3.0 mM): EDTA (0.0558 g) was dissolved in water and the volume was made up to 50 ml with double distilled water.
3. Hydrogen peroxide (1.5 mM): 16 µl of standard hydrogen peroxide (30%) was dissolved in water and the volume was made up to 100 ml with double distilled water.
4. Phosphate buffer (100 mM, pH 7.0)

   Sol A: Potassium dihydrogen phosphate 6.80 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

   Sol B: Di-potassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

   Buffer solution was prepared by mixing 39 ml of sol. A and 61 ml of sol. B, and final pH was adjusted with the help of pH meter

**Enzyme assay**

The reaction mixture (3 ml) contained 50 mM potassium phosphate buffer (pH 7.0) (1.5 ml of 100 mM), 0.5 mM ascorbic acid (0.5 ml of 3.0 mM), 0.1 mM EDTA (0.1 ml of 3.0 mM), 0.1 mM $H_2O_2$ (0.2/0.6 ml of 1.5 mM), 0.1 ml enzyme and water 0.6 ml (final volume of 3.0 ml). Initiation of the reaction was done by adding hydrogen peroxide (0.2 ml). Reduction in the optical density for 1 minute was recorded at 290

nm in an UV-visible spectrophotometer (Dynamica, Halo DB 20, USA). The amount of enzyme necessary to decompose 1 μ mol ascorbate per mg protein was considered as one enzyme unit and is expressed as U mg $protein^{-1}$.

### 3.16.3 Catalase (EC 1.11.1.6)

Catalase enzyme activity was assayed by measuring the disappearance of $H_2O_2$ according to Aebi (1984).

**Reagents**

1. Phosphate buffer (0.1 M, pH 7.0)

**Sol A:** Potassium dihydrogen phosphate 6.8 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

**Sol B:** Di-potassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500 ml with double distilled water.

Buffer was prepared by mixing 39 ml of solution A and 61 ml of solution B, and final pH was adjusted.

2. Hydrogen Peroxide (75 mM): A solution of 75 mM $H_2O_2$ was prepared by dissolving 775 μl of 30% $H_2O_2$ in 100 ml of double distilled water.

**Enzyme assay**

Assay mixture (3 ml) contained 0.5 ml of 75 mM $H_2O_2$ and 1.5 ml of 0.1 M phosphate buffer (pH 7) in cuvette. The reaction was started by the addition of enzyme extract (50 μl). Decrease in absorbance was observed for 1 min in a UV-visible spectrophotometer at 240 nm (Dynamica, Halo DB 20, USA). The concentration of $H_2O_2$ (initial and final) was estimated and the catalase activity was finally calculated by estimating the amount of $H_2O_2$ reduced. Observations were expressed as units based on the amount of enzyme required to decompose 1 μ mole $H_2O_2$ per milligram protein and expressed as U mg $protein^{-1}$.

### 3.16.4 Proline content

The proline content in the cyanobacterial cells was measured by the method of Bates *et al.,* (1973).

**Reagents**

1. Sulfosalicylic acid: 3% aqueous solution.
2. Acid ninhydrin: 2.48 g ninhydrin was dissolved in 60 ml glacial acetic acid and 40 ml of 6N phosphoric acid, kept in 4 ºC.
3. Toluene
4. Glacial acetic acid

**Assay**

Cyanobacterial pellets were homogenized with 3% sulfosalicylic acid (10 ml). The homogenate was filtered using Whatman 42 paper. The suspension (2 ml) was then transferred to test tubes and mixed with acid ninhydrin (2 ml) and glacial acetic acid (2 ml). The suspension was kept at 100 ºC for 60 minutes. Tubes were taken out from water bath, kept immediately in ice to terminate the reaction. 4 ml of toluene was pipette to the mixture and mixed vigorously for 15-20 sec. Optical density of the chromophore from the toluene phase was recorded at 520 nm in UV-visible spectrophotometer (Dynamica, Halo DB 20, USA). A reagent blank was also made and using L-proline a standard curve was plotted.

**3.17 Statistical analysis**

The experiments have been conducted with samples in triplicate and the experimental data was statistically analysed using One way analysis of variance (ANOVA). F-test was used for comparing the significance of treatment means (Gomez and Gomez, 1984). The critical difference ($P=0.01$) was worked out with respect to treatment means to evaluate differences between treatment means.

*Chapter 4*

# RESULTS

## 4.1 Determination of maximum tolerance limit to NaCl and $Na_2SO_4$ by the cyanobacterium *Anabaena doliolum*

In order to determine the maximum tolerance limit to NaCl and $Na_2SO_4$ by the cyanobacterium *Anabaena doliolum*, the cells were subjected to different concentrations of NaCl and $Na_2SO_4$ (0-250 mM) individually for 8 days. **Plate 1a & b** depicts the inhibition in growth of *A. doliolum* subjected to various concentrations of NaCl and $Na_2SO_4$. The cells not receiving any salt treatment served as control. Growth was estimated in terms of chlorophyll and it was observed that the cyanobacterium was able to tolerate NaCl upto 150 mM whereas, it tolerated $Na_2SO_4$ upto 100 mM **(Fig. 4.1 a & b).** NaCl above 150 mM was found to be inhibitory whereas, $Na_2SO_4$ above 100 mM was inhibitory for the growth of *A. doliolum*. Based on these results, NaCl (150 mM) and $Na_2SO_4$ (100 mM) were used for further experiments on the individual as well as combined effect of NaCl and $Na_2SO_4$.

## 4.2 Effect of NaCl and $Na_2SO_4$ on the growth (dry weight) of the cyanobacterium *Anabaena doliolum*

**Fig. 4.2** depicts the dry weight as an index of growth of the cyanobacterium *Anabaena doliolum* in response to salinity exposure. Increase in the dry weight was studied as a measure of growth. **Plate 2** shows the inhibitory effect of individual as well as combined exposure to NaCl, $Na_2SO_4$ and a mixture of NaCl and $Na_2SO_4$. Significant inhibition in growth was observed both in case of NaCl ($T_2$) and $Na_2SO_4$ ($T_3$) induced salinity. However, combined exposure to NaCl and $Na_2SO_4$ ($T_4$) resulted in further inhibition in dry weight. The dry weight accumulation decreased by 77% in response to NaCl treatment at 144 hours after exposure whereas, the decrease in dry weight due to $Na_2SO_4$ was 80%. Combined exposure resulted in 96% decrease. Therefore, it was observed that all the salinities (NaCl, $Na_2SO_4$ and a mixture of NaCl and $Na_2SO_4$) inhibited the growth. The data was statistically analysed and it showed significant differences between the chloride and sulphate induced salinity ($P<0.01$) treatments $T_2$, $T_3$ and $T_4$ (**Table 4.1**).

## 4.3 Effect of NaCl and $Na_2SO_4$ on the soluble protein content of the cyanobacterium *Anabaena doliolum*.

**Fig. 4.3** shows the protein content from *A. doliolum* cells exposed to salinity. The soluble protein content at $T_2$ (150 mM NaCl) was 21.23% of the control ($T_1$) at 144 hours after incubation. At $T_3$ (100 mM $Na_2SO_4$) the soluble protein content was 34.51% of the control. It was however, observed that the protein content decreased by 41.59% in $T_4$ (150 mM NaCl + 100 mM $Na_2SO_4$). The protein content also showed a declining trend as in the case of growth. The data showed significant differences in the protein content between chloride and sulphate induced salinity ($P<0.01$, **Table 4.1**).

## 4.4 Effect of NaCl and $Na_2SO_4$ on the pigment profile of the cyanobacterium *Anabaena doliolum*

The amount of chlorophyll is generally considered as an index of biomass accumulation. In the present study, the salinity treatment $T_2$ decreased chlorophyll content by 68.69% at 144 hours after salinity exposure (**Fig. 4.4**). However, the decrease (60.86%) was not severe in case of treatment $T_3$. Combined exposure to NaCl and $Na_2SO_4$ in the treatment $T_4$ resulted in severe and drastic reduction in the chlorophyll content. At $T_4$, an inhibition of 92.73% in chlorophyll content was noticed. Similarly, the phycocyanin, allophycocyanin and phycoerythrin also exhibited inhibition due to individual as well as mixture of NaCl and $Na_2SO_4$ treatment (**Fig. 4.5, 4.6 and 4.7**). The phycocyanin content was found to decrease by 82.53%, 74.76% and 94.96% at $T_2$, $T_3$ and $T_4$ respectively as compared to the unexposed control. The observed reduction in the allophycocyanin content was 83.89%, 76.42% and 93.84% at identical level of salinity treatment. The inhibition in the phycoerythrin content was in the range of 80.95 to 91.69% at various treatments. The carotenoid content of the cyanobacterium, although important as an anti-oxidant under stress conditions was found to decrease in response to salinity treatment. In the percentage investigation, it was observed that, the carotenoid content decreased by 64.6%, 66.32% and 81.02% respectively, in response to salinity treatments (**Fig. 4.8**). In this case also the inhibition was found to be severe when the growth medium was supplemented with a mixture of NaCl and $Na_2SO_4$. The data on the pigment content was analysed statistically and the analysis showed significant differences in the various pigments in the cyanobacterium *A. doliolum* in response to chloride and sulphate induced salinity as well a combination of chloride and sulphate ($P<0.01$, **Table 4.1**).

## 4.5 Effect of NaCl and $Na_2SO_4$ on the sugar content of the cyanobacterium *Anabaena doliolum*

While most of the cellular constituents decreased in response to salinity, the sugar content was found to increase in the cyanobacterium (**Fig. 4.9**). The level of cellular sugar content increased with respect to time and attained a maximum limit at 144 hour of salt exposure. Maximum accumulation of sugars was found in response to $T_2$ followed by $T_3$ and $T_4$. The highest sugar level was observed at 150 mM NaCl (19.83% of control) followed by $Na_2SO_4$ (5.6% of control). A combination of NaCl and $Na_2SO_4$ resulted in accumulation of less amount of sugar in *A. doliloum* as compared to individual exposure to salinity. Statistical analysis of the data showed significant and positive influence on the sugar levels in due to chloride and sulphate mediated salinity ($P<0.01$, **Table 4.1**).

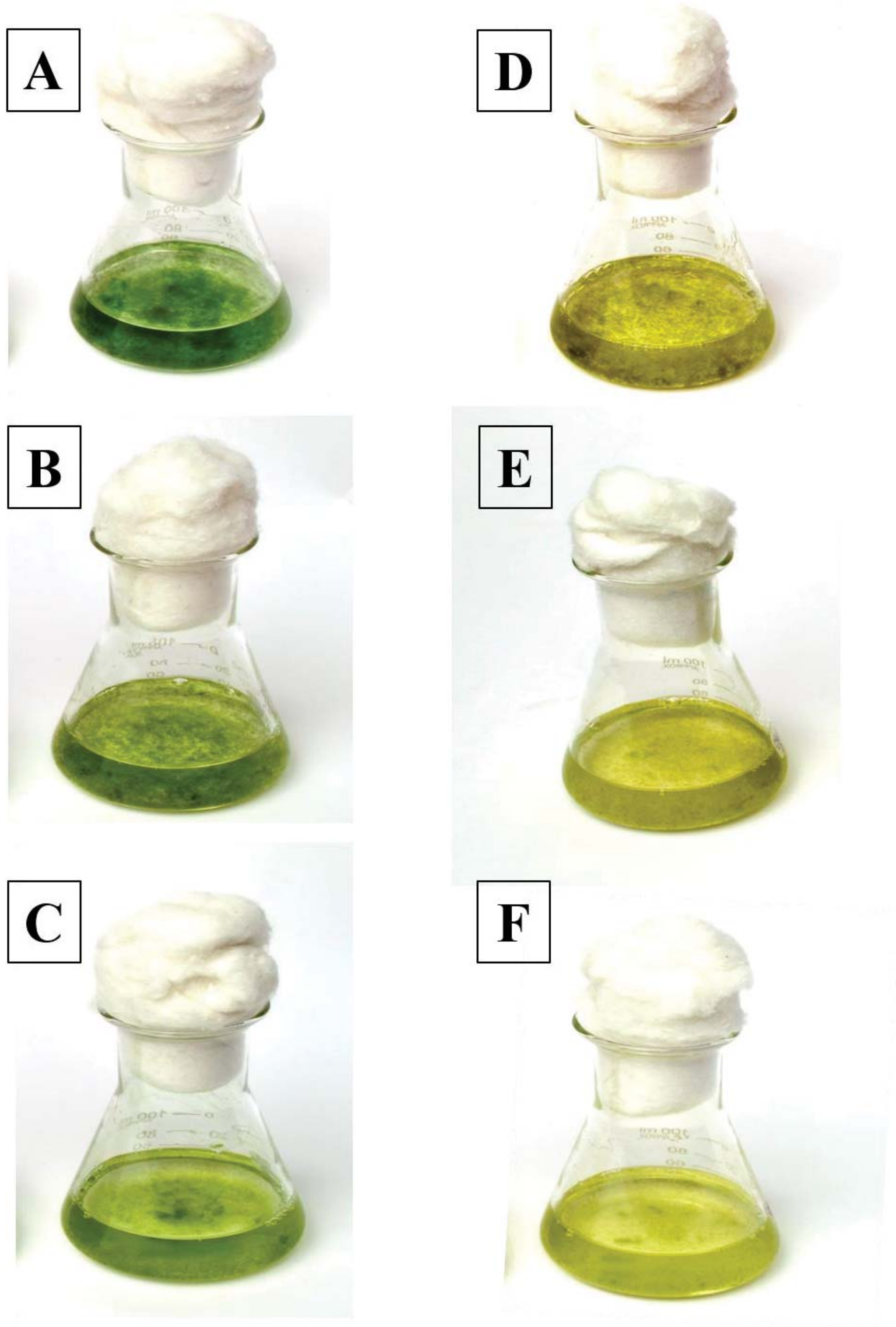



**Plate No. 1a. Inhibition in growth of *A. doliolum* exposed to different concentrations of NaCl**

A: 0 mM, B: 50 mM, C: 100 mM, D: 150 mM, E: 200 mM, F: 250 mM

**Plate No. 1b. Inhibition in growth of *A. doliolum* exposed to different concentrations of $Na_2SO_4$**

A: 0 mM, B: 50 mM, C: 100 mM, D: 150 mM, E: 200 mM, F: 250 mM

**Plate No. 2. The inhibitory effect of NaCl, $Na_2SO_4$ and a mixture of NaCl and $Na_2SO_4$ on *A. doliolum***

$T_1$: Control, $T_2$ : 150 mM NaCl, $T_3$: 100 mM $Na_2SO_4$, $T_4$: 150 mM NaCl+ 100 mM $Na_2SO_4$

**Table 4.1. Impact of NaCl and $Na_2SO_4$ salinity on growth, pigments and cellular constituents of the cyanobacterium *Anabaena doliolum* at 144 h.**

| | Parameters | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Treatments** | **Dry weight (mg ml$^{-1}$)** | **Chlorophyll (mg ml$^{-1}$)** | **Carotenoids (µg ml$^{-1}$)** | **Phycocyanin (µg ml$^{-1}$)** | **Allophycocyanin (µg ml$^{-1}$)** | **Phycoerythrin (µg ml$^{-1}$)** | **Protein (mg g dry weight$^{-1}$)** | **Sugar (mg g dry weight$^{-1}$)** |
| **Control** | 0.037±0.003 | 1.177±0.015 | 0.631±0.011 | 0.094±0.001 | 0.097±0.001 | 0.039±0.002 | 22.400± 0.116 | 178.750± 0.789 |
| **NaCl** | 0.007±0.000 | 0.366±0.008 | 0.222±0.003 | 0.017±0.000 | 0.016±0.000 | 0.007±0.000 | 17.433± 0.203 | 222.496± 0.610 |
| **$Na_2SO_4$** | 0.008±0.000 | 0.453±0.019 | 0.218±0.006 | 0.025±0.001 | 0.023±0.001 | 0.011±0.001 | 14.500± 0.173 | 190.263± 0.704 |
| **NaCl + $Na_2SO_4$** | 0.001±0.000 | 0.084±0.000 | 0.119±0.001 | 0.006±0.001 | 0.006±0.000 | 0.004±0.000 | 13.167± 0.203 | 183.440± 0.718 |
| **F value** | 101.069 | 1388.124 | 1321.978 | 3544.596 | 4121.058 | 335.037 | 533.197 | 778.847 |
| **P value** | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| **SE(m)** | 0.002 | 0.012 | 0.006 | 0.001 | 0.001 | 0.001 | 0.177 | 0.708 |
| **CD** | 0.005 | 0.041 | 0.021 | 0.002 | 0.002 | 0.003 | 0.587 | 2.343 |

Data are means±standard error of three replicates (n=3). Data shows significant difference at P<0.01 level.

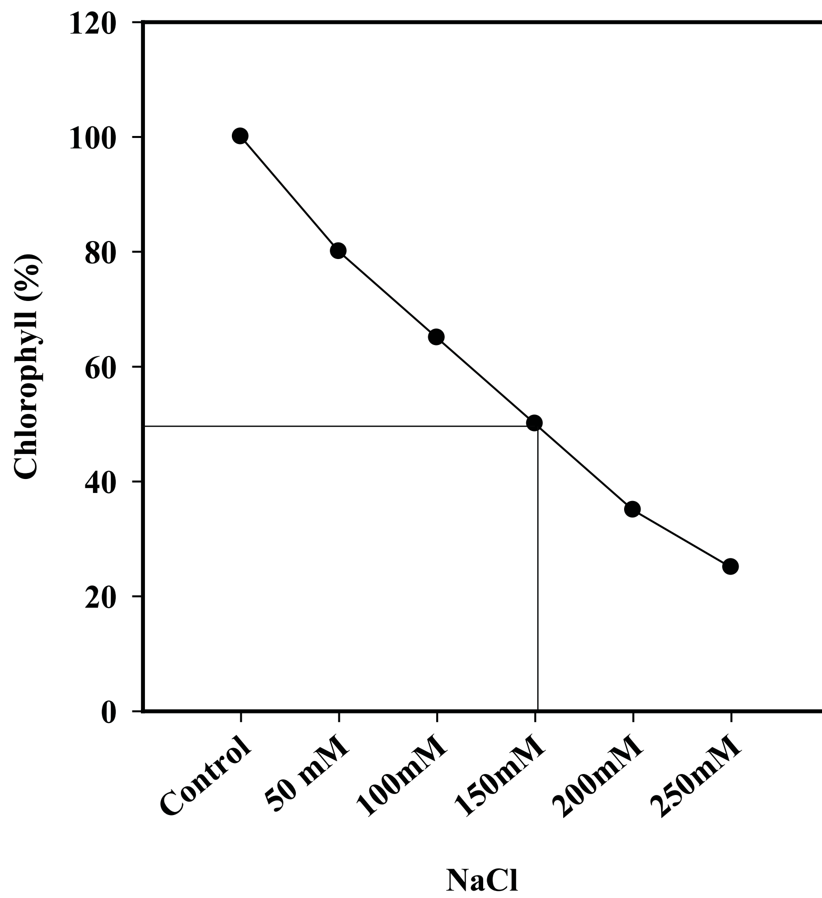



**Fig. 4.1a. Maximal inhibitory concentration of NaCl on the growth of *A. doliolum* estimated in terms of chlorophyll content.**

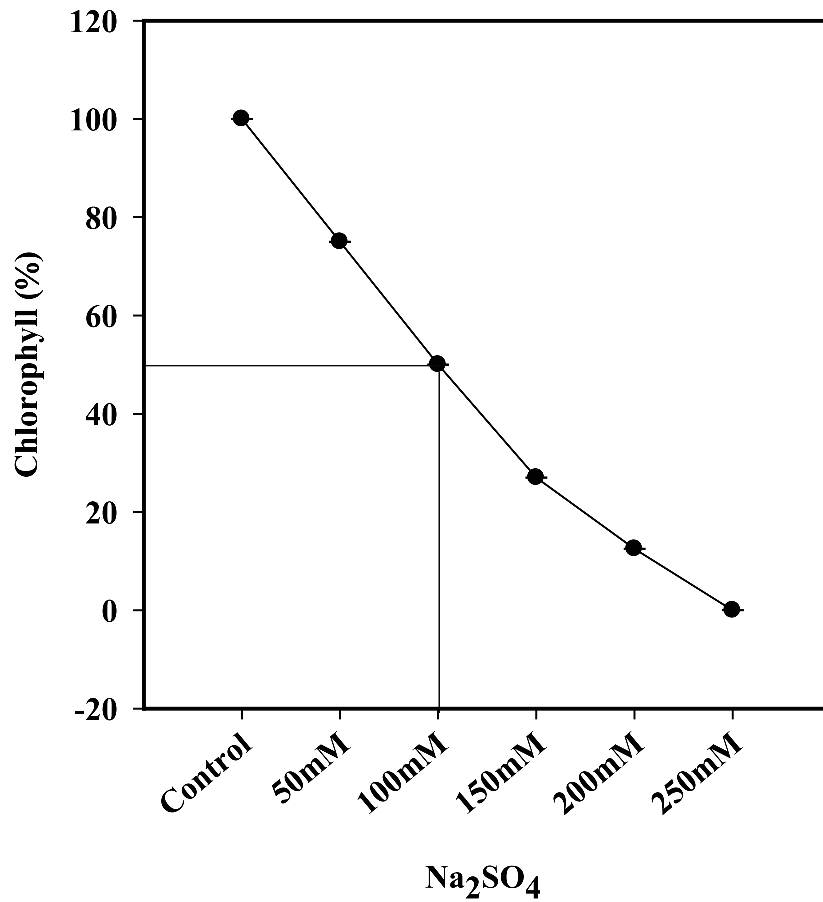



**Fig. 4.1b. Maximal inhibitory concentration of $Na_2SO_4$ on the growth of *A. doliolum* estimated in terms of chlorophyll content.**

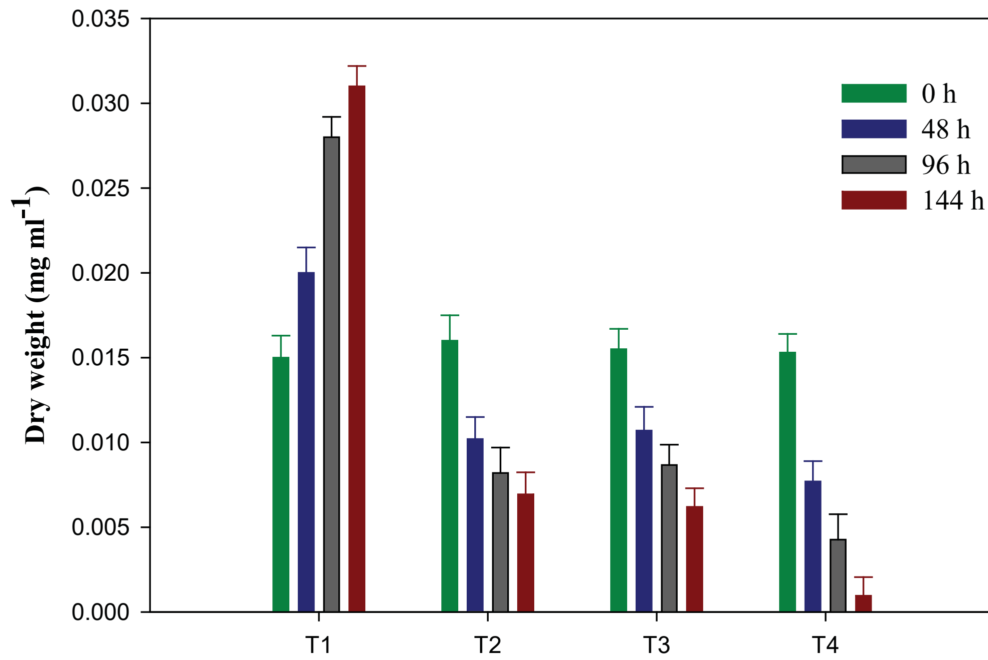



**Fig. 4.2. Effect of salinity on dry weight content of the cyanobacterium *A. doliolum* in response to NaCl and $Na_2SO_4$.**

**$T_1$**: Control, **$T_2$**: 150 mM NaCl, **$T_3$**: 100 mM $Na_2SO_4$, **$T_4$**: 150 mM NaCl + 100 mM $Na_2SO_4$

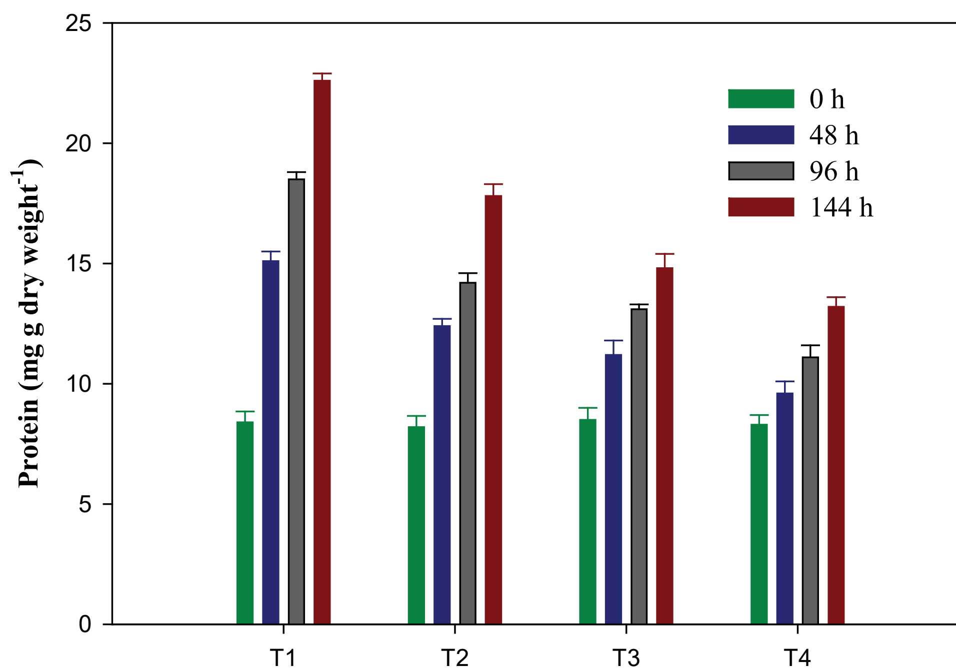



**Fig. 4.3. Response of salinity on the protein content of the cyanobacterium *A. doliolum*.**

**T1**: Control, **T2**: 150 mM NaCl, **T3**: 100 mM $Na_2SO_4$, **T4**: 150 mM NaCl + 100 mM $Na_2SO_4$

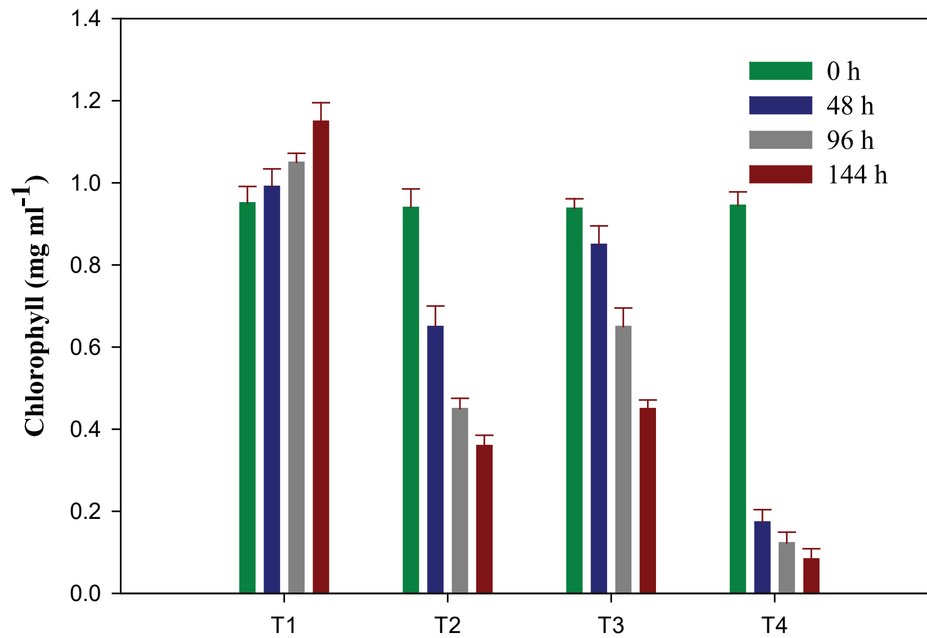



**Fig. 4.4. Effect of salinity on chlorophyll content of the cyanobacterium *A. doliolum*.**

**$T_1$**: Control, **$T_2$**: 150 mM NaCl, **$T_3$**: 100 mM $Na_2SO_4$, **$T_4$**: 150 mM NaCl + 100 mM $Na_2SO_4$

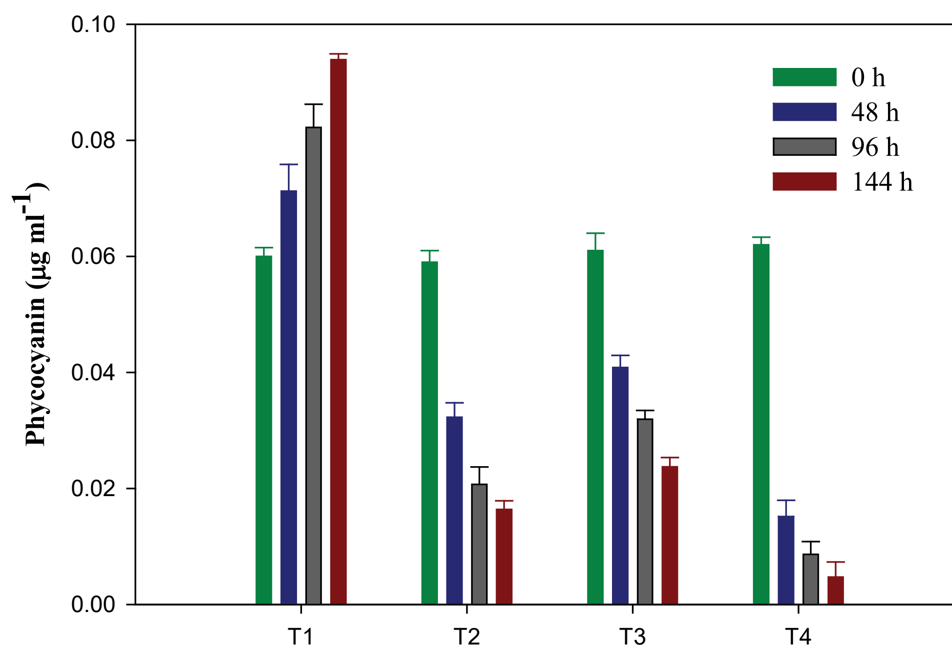



**Fig. 4.5. Effect of salinity on the phycocyanin content of the cyanobacterium *A. doliolum*.**

**T1**: Control, **T2**: 150 mM NaCl, **T3**: 100 mM $Na_2SO_4$, **T4**: 150 mM NaCl + 100 mM $Na_2SO_4$

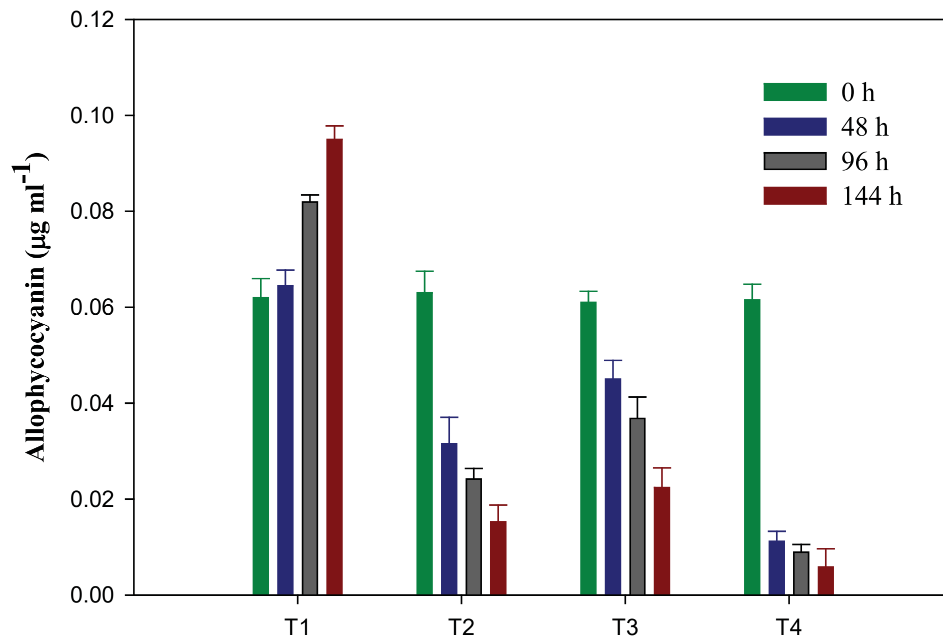



**Fig. 4.6. Effect of salinity on the allophycocyanin content of the cyanobacterium *A. doliolum*.**

**T1**: Control, **T2**: 150 mM NaCl, **T3**: 100 mM $Na_2SO_4$, **T4**: 150 mM NaCl + 100 mM $Na_2SO_4$

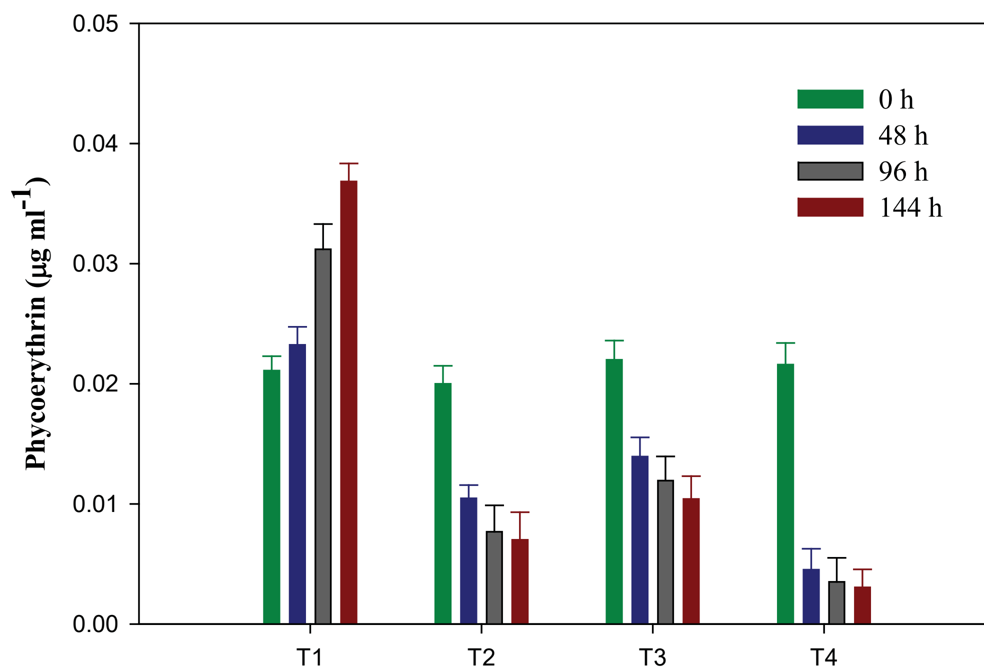



**Fig. 4.7. Effect of salinity on the phycoerythrin content of the cyanobacterium *A. doliolum*.**

**$T_1$**: Control, **$T_2$**: 150 mM NaCl, **$T_3$**: 100 mM $Na_2SO_4$, **$T_4$**: 150 mM NaCl + 100 mM $Na_2SO_4$

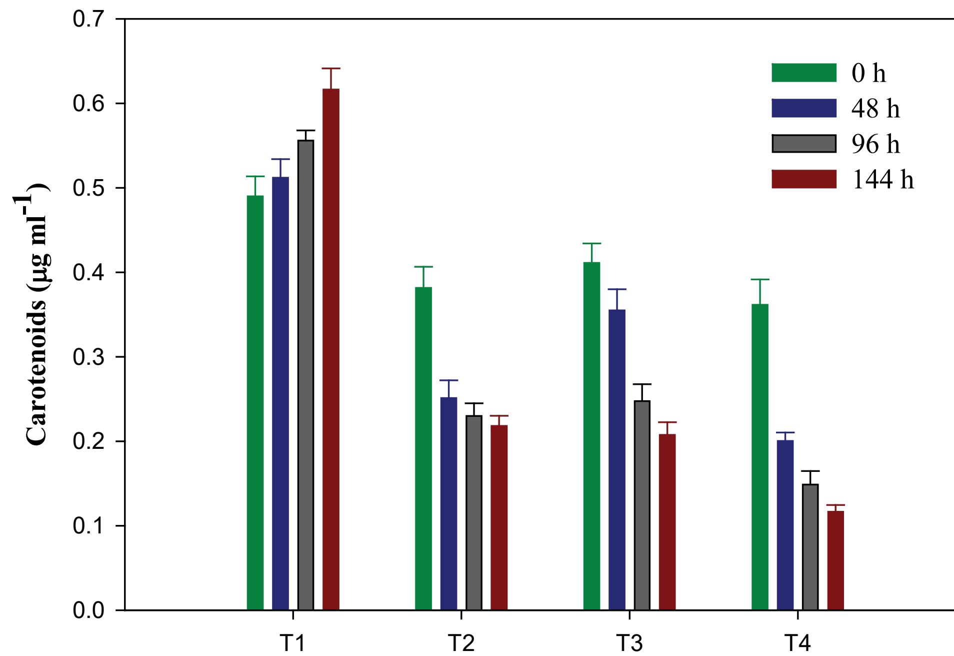



**Fig. 4.8. Effect of salinity on the carotenoid content of the cyanobacterium *A. doliolum.***

**T1**: Control, **T2**: 150 mM NaCl, **T3**: 100 mM $Na_2SO_4$, **T4**: 150 mM NaCl + 100 mM $Na_2SO_4$

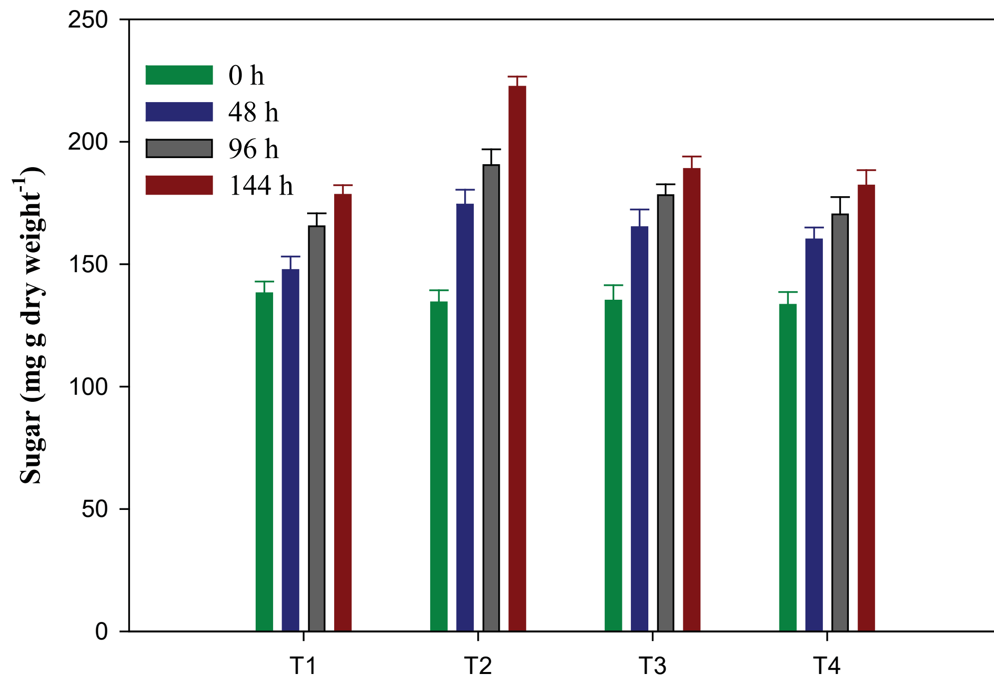



**Fig. 4.9. Effect of salinity on the sugar content of the cyanobacterium *A. doliolum*.**

**T1**: Control, **T2**: 150 mM NaCl, **T3**: 100 mM $Na_2SO_4$, **T4**: 150 mM NaCl + 100 mM $Na_2SO_4$

## 4.6 Effect of NaCl and $Na_2SO_4$ on the intracellular ion concentration ($Na^+$, $K^+$ and $Ca^{2+}$) of the cyanobacterium *Anabaena doliolum*

The present study revealed that salinity (150 mM NaCl) enhanced the intracellular sodium levels significantly in the cyanobacterium *A. doliolum* (**Fig. 4.10**). Accumulation of intracellular $Na^+$ was low in response to 100 mM $Na_2SO_4$ treatment. On the contrary, in the salt mixture (NaCl + $Na_2SO_4$) the cells were found to drastically increase the intracellular $Na^+$ content. The intracellular $K^+$ content although increased in response to NaCl and $Na_2SO_4$, did not show much significant increase in relation to NaCl and $Na_2SO_4$. Salinity resulted in higher cellular $Ca^{2+}$ content in the cyanobacterium *A. doliolum*. The $Ca^{2+}$ level in the NaCl treated cells increased by 43.18% whereas, the $Ca^{2+}$ content in cells exposed to $Na_2SO_4$ increased by 60.93%. However, increase in the cellular $Ca^{2+}$ content in the cyanobacterial cells exposed to a mixture of NaCl and $Na_2SO_4$ showed 45.65% increase. Increase in the cellular $Na^+$, $K^+$ and $Ca^{2+}$ content showed significant relation with the chloride and sulphate treatment as well as their combination ($P<0.01$), **Table 4.2**).

From the results obtained regarding cellular $Na^+$, $K^+$ and $Ca^{2+}$ content in the cyanobacterium *A. doliolum* we worked out the $K^+/Na^+$ and $Ca^{2+}/Na^+$ ratio (Table 4.3). The cells exposed to NaCl in the treatment $T_2$ failed to maintain higher $K^+/Na^+$ ratio. The $K^+/Na^+$ ratio reduced drastically at $T_4$ where the cyanobacterium is exposed to a combination of NaCl and $Na_2SO_4$. Similar trend was also observed in *A. doliolum* with respect to $Ca^{2+}/Na^+$ ratio. Ratio of $Ca^{2+}/Na^+$ was 0.686 at $T_1$ and the ratio decreased to 0.40 at $T_2$ and to 0.267 at $T_4$. However, at $T_3$, where the cyanobacterium was exposed to $Na_2SO_4$, the $K^+/Na^+$ and $Ca^{2+}/Na^+$ ratio was found to be almost at par with the control despite reduction in growth and cellular constituents. Exposure of the cyanobacterium to chloride, sulphate and a combination of chloride and sulphate showed statistically significant differences in the intracellular $K^+/Na^+$ and $Ca^{2+}/Na^+$ ratio ($P<0.01$, **Table 4.3**).

**Table 4.2. Salinity induced changes in the intracellular ion concentration in *A. doliolum* at 144 h after NaCl and $Na_2SO_4$ exposure.**

| Treatments | $Na^+$ (μ mol mg protein$^{-1}$) | $K^+$ (μ mol mg protein$^{-1}$) | $Ca^{2+}$ (μ mol mg protein$^{-1}$) |
|---|---|---|---|
| **Control** | 1.810±0.006 | 0.477±0.005 | 1.250±0.017 |
| **NaCl** | 5.610±0.061 | 0.914±0.007 | 2.243±0.026 |
| **$Na_2SO_4$** | 4.813±0.041 | 1.147±0.024 | 3.237±0.020 |
| **NaCl + $Na_2SO_4$** | 8.500±0.058 | 0.828±0.018 | 2.343±0.026 |
| **F value** | 3461.413 | 316.744 | 1277.681 |
| **P value** | <0.01 | <0.01 | <0.01 |
| **SE(m)** | 0.047 | 0.016 | 0.023 |
| **CD** | 0.155 | 0.052 | 0.075 |

Data are means±standard error of three replicates (n=3). Data shows significant difference at P<0.01 level.

**Table 4.3. Ratio of ions ($K^+/Na^+$ and $Ca^{2+}/Na^+$) in *A. doliolum* in response to NaCl and $Na_2SO_4$ exposure.**

| Treatments | $K^+/Na^+$ ratio | $Ca^{2+}/Na^+$ ratio |
|---|---|---|
| **Control** | 0.266 | 0.686 |
| **NaCl** | 0.163 | 0.40 |
| **$Na_2SO_4$** | 0.229 | 0.66 |
| **NaCl + $Na_2SO_4$** | 0.093 | 0.267 |

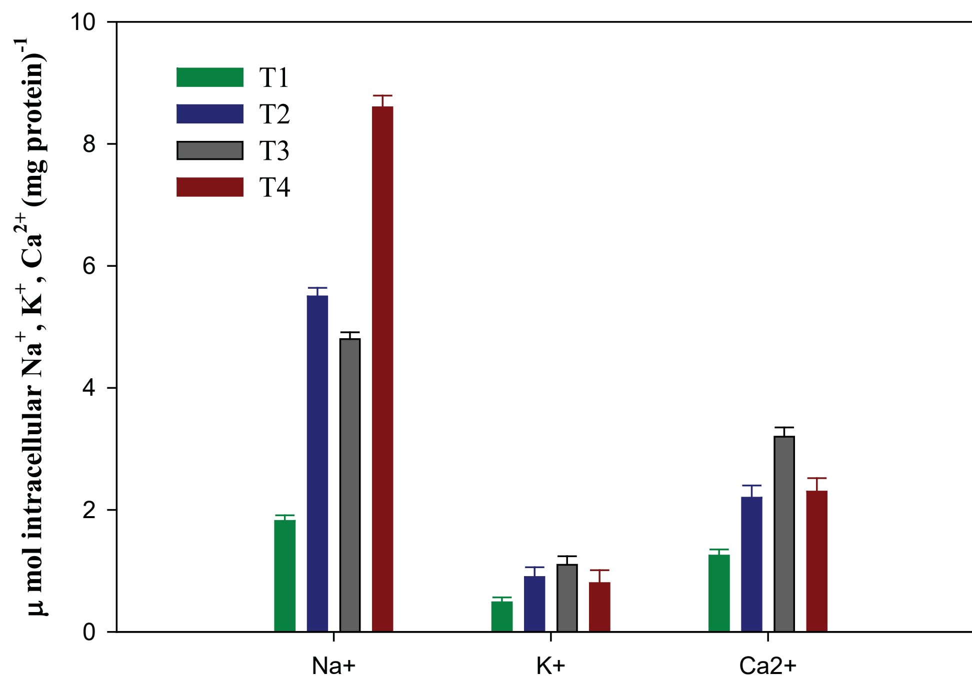



**Fig. 4.10. Effect of salinity on the intracellular ion content of the cyanobacterium *A. doliolum*.**

**$T_1$**: Control, **$T_2$**: 150 mM NaCl, **$T_3$**: 100 mM $Na_2SO_4$, **$T_4$**: 150 mM NaCl + 100 mM $Na_2SO_4$

## 4.7 Effect of NaCl and $Na_2SO_4$ on the heterocyst frequency and average filament length of the cyanobacterium *Anabaena doliolum*

Effect of salinity on the average filament length and heterocyst frequency of the cyanobacterium *A. doliolum* in response to alterations caused by salinity was investigated (**Fig. 4.11**). The average filament length (0.313) was maximum at $T_1$ (control) followed by $T_3$ (0.256), $T_2$ (0.217) and $T_4$ (0.137). The heterocyst frequency was maximum in case of the control (8.43%). However, the heterocyst frequency at $T_2$ and $T_3$ were 5.81 and 6.4% respectively. Maximum decline in the heterocyst frequency was recorded at $T_4$ (4.1%). Analysis of the data showed statistically significant differences in the average filament length and heterocyst frequency in response to chloride and sulphate induced salinity and their interaction ($P<0.01$, **Table 4.4**).

## 4.8 Effect of NaCl and $Na_2SO_4$ on the nitrogen assimilation enzymes of the cyanobacterium *Anabaena doliolum*

Effect of salinity on the enzymes of nitrogen assimilation such as nitrogenase, nitrate reductase and glutamine synthetase was studied (**Fig. 4.12, 4.13 & 4.14**). The nitrate reductase decreased significantly in the cyanobacterium *A. doliolum* in response to individual as well as combined salinity treatment. The activity of ammonia assimilation enzyme, glutamine synthetase also showed significant inhibition in its activity. At $T_1$, the nitrogenase activity observed on 144 hours after salinity treatment was 62.95 n mol $C_2H_4$ mg $chl^{-1}$ $hr^{-1}$. The activity of nitrogenise drastically reduced to 12.4, 15.1 and 9.2 n mol $C_2H_4$ mg $chl^{-1}$ $hr^{-1}$ at treatments $T_2$, $T_3$ and $T_4$. The results conclusively showed that salinity adversely affected the activity of nitrogen assimilation enzymes in the cyanobacterium *A. doliolum*. The activity of nitrogen assimilation enzymes such as nitrogenase, nitrate reductase and glutamine synthetase enzymes showed significant statistical differences with respect to chloride and sulphate. Similar results were observed in response to a combination of chloride and sulphate ($P<0.01$, **Table 4.4**).

**Table 4.4. Heterocyst frequency, average filament length and the activity of nitrogen assimilation enzymes in *A. doliolum* due to NaCl and $Na_2SO_4$ exposure at 144 h.**

| | Parameters | | | | |
|---|---|---|---|---|---|
| **Treatments** | **Heterocyst frequency (%)** | **Average filament length** | **Nitrogenase (n mol $C_2H_4$ mg $chl^{-1}$ $h^{-1}$)** | **Nitrate reductase (µ mol mg $protein^{-1}$)** | **Glutamine synthetase (µ mol mg $protein^{-1}$)** |
| **Control** | 8.440±0.032 | 0.317±0.002 | 63.493±0.344 | 8.600±0.058 | 0.189±0.003 |
| **NaCl** | 5.800±0.032 | 0.216±0.001 | 13.033±0.328 | 1.867±0.080 | 0.040±0.000 |
| **$Na_2SO_4$** | 6.520±0.064 | 0.253±0.002 | 15.600±0.321 | 2.383±0.080 | 0.058±0.002 |
| **NaCl + $Na_2SO_4$** | 4.160±0.031 | 0.133±0.002 | 8.652±0.318 | 0.911±0.027 | 0.015±0.000 |
| **F value** | 1764.219 | 1391.654 | 6136.902 | 2893.046 | 2196.428 |
| **P value** | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| **SE(m)** | 0.042 | 0.002 | 0.328 | 0.065 | 0.002 |
| **CD** | 0.140 | 0.007 | 1.086 | 0.215 | 0.006 |

Data are means±standard error of three replicates (n=3). Data shows significant difference at P<0.01 level.

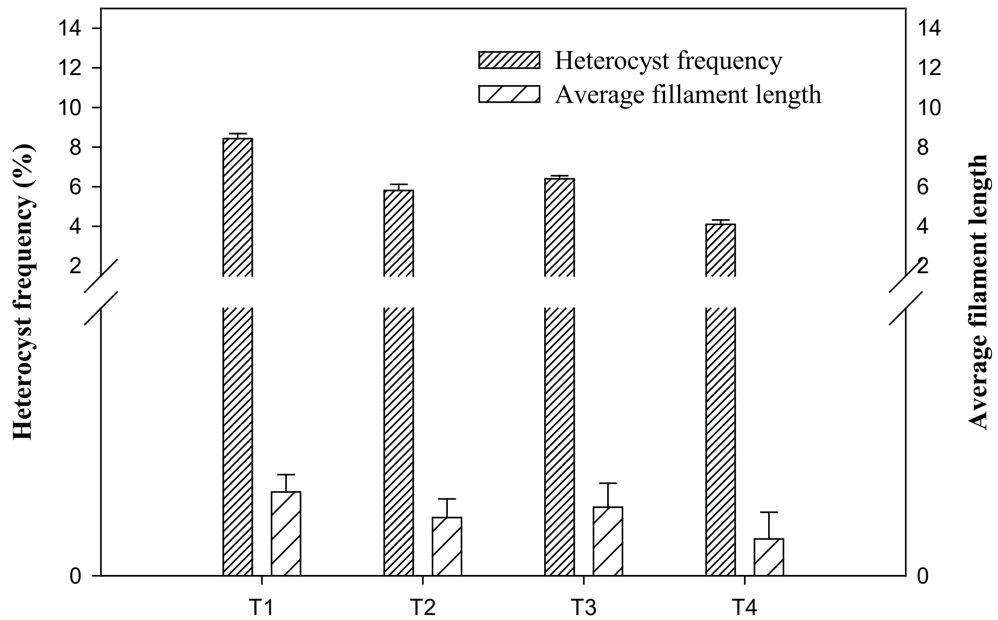



**Fig. 4.11. Effect of salinity on the heterocyst frequency and average filament length of the cyanobacterium *A. doliolum*.**

**T1**: Control, **T2**: 150 mM NaCl, **T3**: 100 mM $Na_2SO_4$, **T4**: 150 mM NaCl + 100 mM $Na_2SO_4$

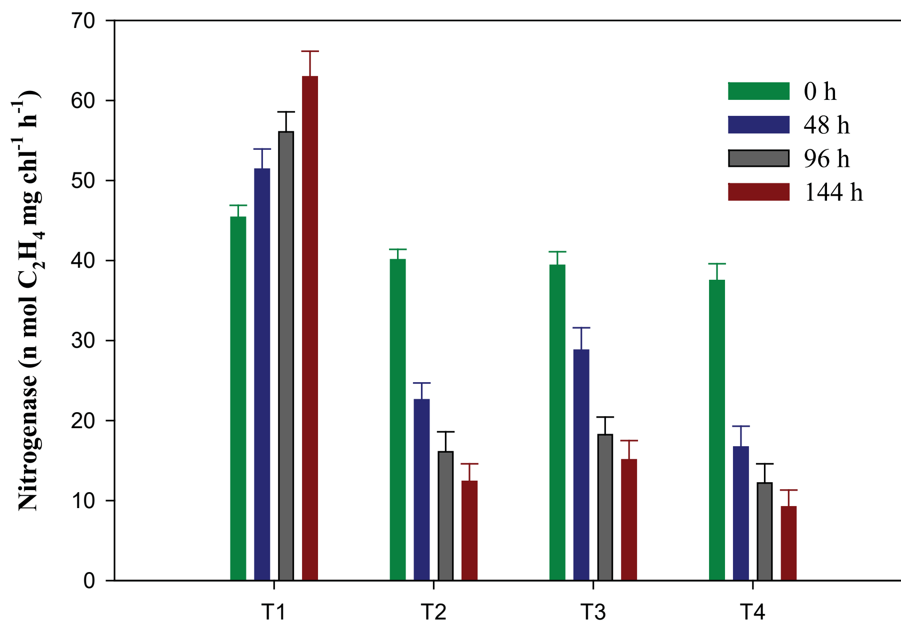



**Fig. 4.12. Effect of salinity on the nitrogenase activity of the cyanobacterium *A. doliolum*.**

**$T_1$**: Control, **$T_2$**: 150 mM NaCl, **$T_3$**: 100 mM $Na_2SO_4$, **$T_4$**: 150 mM NaCl + 100 mM $Na_2SO_4$

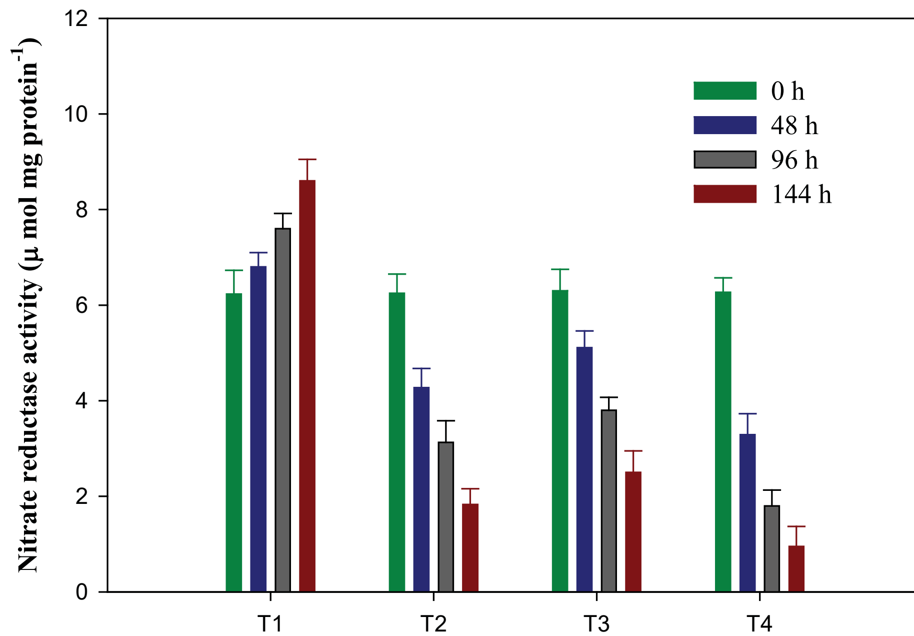



**Fig. 4.13. Effect of salinity on the nitrate reductase activity of the cyanobacterium *A. doliolum*.**

**T1**: Control, **T2**: 150 mM NaCl, **T3**: 100 mM $Na_2SO_4$, **T4**: 150 mM NaCl + 100 mM $Na_2SO_4$

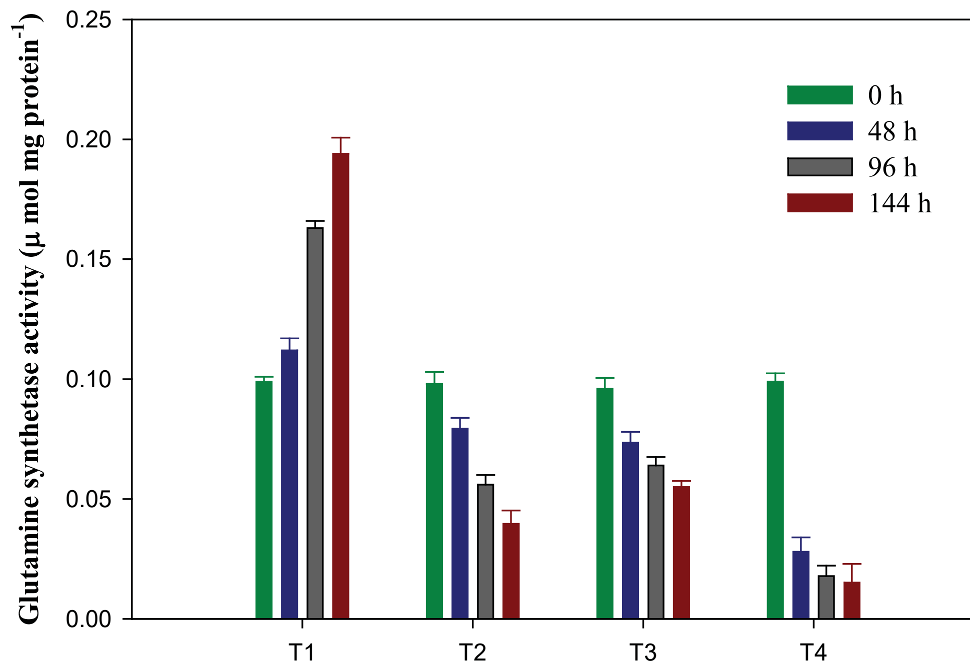



**Fig. 4.14. Effect of salinity on the glutamine synthetase activity of the cyanobacterium *A. doliolum*.**

**$T_1$**: Control, **$T_2$**: 150 mM NaCl, **$T_3$**: 100 mM $Na_2SO_4$, **$T_4$**: 150 mM NaCl + 100 mM $Na_2SO_4$

## 4.9 Lipid peroxidation activity in the cyanobacterium *Anabaena doliolum* in response to NaCl and $Na_2SO_4$ exposure

Exposure of *A. doliolum* to differential salt treatment resulted in the acuumulation of lipid peroxidation products more in a mixture of NaCl and $Na_2SO_4$ than NaCl and $Na_2SO_4$ alone. Significant increase in the lipid peroxidation product occurred from the second day (48 h) the cells are exposed to various salinity treatments (**Fig. 4.15**). Significant accumulation of lipid peroxidation products was also observed at 96 and 144 hours. Accumulation of lipid peroxidation products was found to be statistically significant in response to chloride and sulphate induced salinity as well as their combionation ($P<0.01$, Table **4.5**).

## 4.10 Effect of NaCl and $Na_2SO_4$ on the enzymatic and non-enzymatic antioxidant molecules of the cyanobacterium *Anabaena doliolum*

The profile of the antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase and catalase were investigated in cyanobacterium *A. doliolum* exposed to salinity (**Fig. 4.16, 4.17 & 4.18**). The activity of superoxide dismutase was at its peak in the treatment $T_4$ on 144 hours after exposure to salinity. It was also observed that the activity increased in response to individual treatment of NaCl (35.23%) and $Na_2SO_4$ (26.08%). Similar results have been observed with respect to the ascorbate peroxidise activity also. The activity increased by 57.83% and 50.52% at $T_2$ and $T_3$ whereas at $T_4$ the increase in the activity was 66.28%. Catalase activity of the cells exposed to $T_4$ increased by 67.87% followed by $T_2$ (64.53%) and $T_3$ (45.52%). Intracellular proline content of the cyanobacterium *A. doliolum* was estimated in response to growth under saline conditions (**Fig. 4.19**). Salinity treatment increased the cellular proline maximally in the treatment $T_4$ (61.84%) followed by $T_2$ (56.63%) and $T_3$ (41.45%). Increase in the activity of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidise, catalase and proline showed significant differences in response to salinity induced by chloride, sulphate and a mixture of these salts ($P<0.01$, **Table 4.5**).

**Table 4.5. Lipid peroxidation, Proline accumulation and the activity of antioxidant enzymes in *A. doliolum* exposed to NaCl and $Na_2SO_4$ at 144 h.**

| | Parameters | | | | |
|---|---|---|---|---|---|
| **Treatments** | **MDA Content (µ mol mg protein$^{-1}$)** | **SOD (U mg protein$^{-1}$)** | **APX (U mg protein$^{-1}$)** | **CAT (U mg protein$^{-1}$)** | **Proline (n mol mg protein$^{-1}$)** |
| **Control** | 3.078±0.027 | 1.246±0.005 | 12.000±0.081 | 5.722±0.062 | 1.234±0.006 |
| **NaCl** | 5.335±0.045 | 1.907±0.030 | 27.777±0.374 | 15.727±0.247 | 2.770±0.028 |
| **$Na_2SO_4$** | 4.035±0.024 | 1.720±0.035 | 24.805±0.273 | 11.423±0.573 | 2.098±0.036 |
| **NaCl + $Na_2SO_4$** | 6.188±0.030 | 2.157±0.030 | 36.143±0.270 | 17.850±0.232 | 3.268±0.033 |
| **F value** | 1783.216 | 198.936 | 1364.037 | 256.803 | 995.774 |
| **P value** | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| **SE(m)** | 0.033 | 0.027 | 0.271 | 0.334 | 0.028 |
| **CD** | 0.108 | 0.090 | 0.898 | 1.107 | 0.092 |

Data are means±standard error of three replicates (n=3). Data shows significant difference at P<0.01 level.

**MDA**: In terms of Thiobarbituric Acid Reactive Substances (TBARS), **SOD**: Super Oxide Dismutase, **APX**: Ascorbate Peroxidase, **CAT**: Catalase

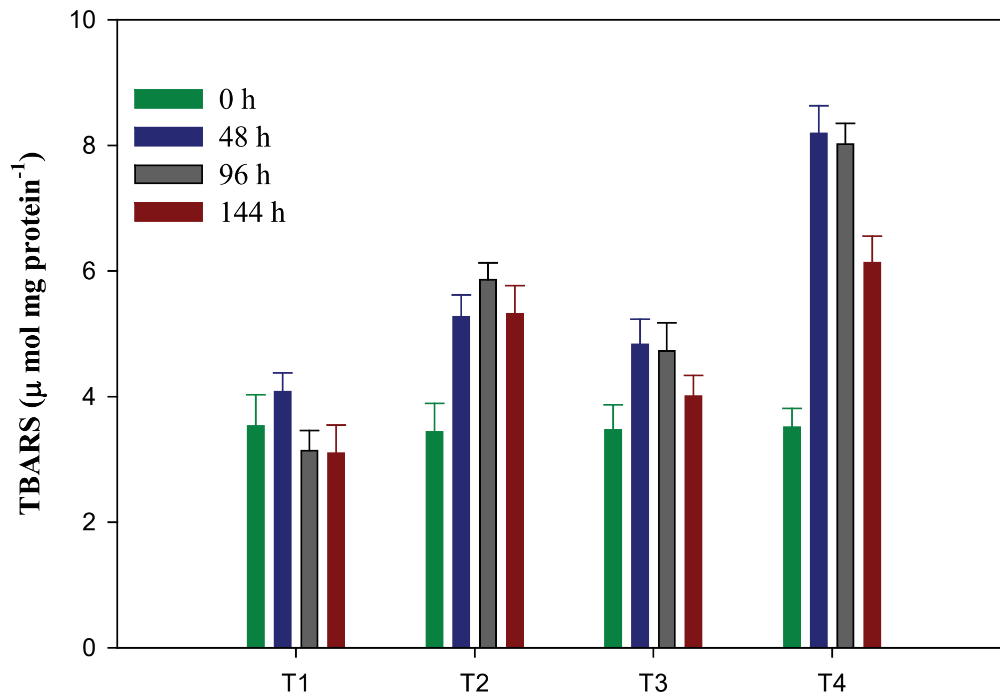



**Fig. 4.15. Effect of salinity on the lipid peroxidation by the cyanobacterium *A. doliolum*.**

**$T_1$**: Control, **$T_2$**: 150 mM NaCl, **$T_3$**: 100 mM $Na_2SO_4$, **$T_4$**: 150 mM NaCl + 100 mM $Na_2SO_4$

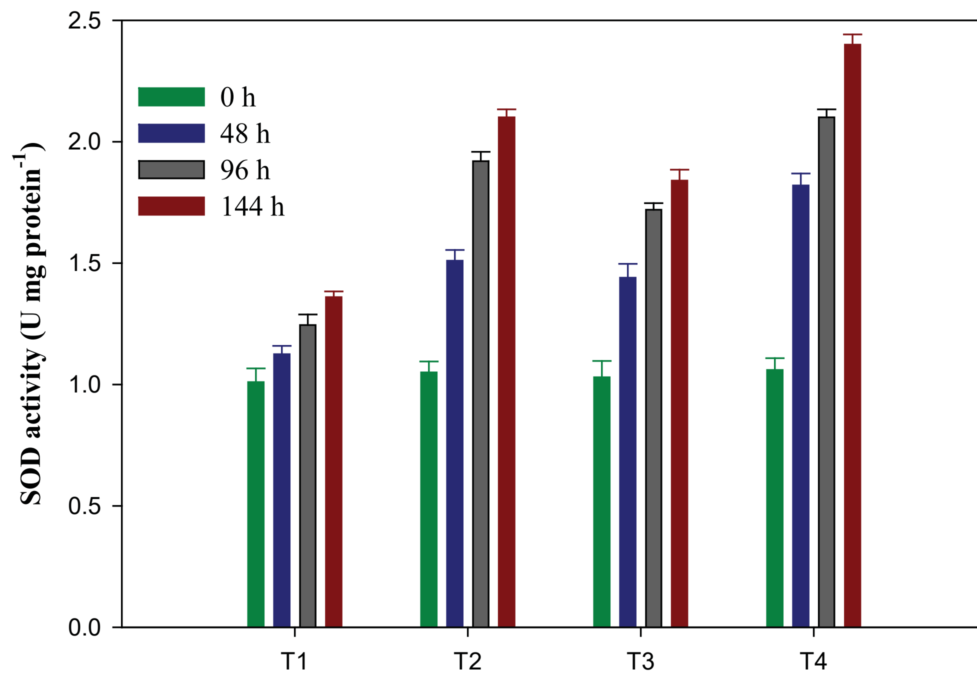



**Fig. 4.16. Effect of salinity on the superoxide dismutase (SOD) activity of the cyanobacterium *A. doliolum*.**

**$T_1$**: Control, **$T_2$**: 150 mM NaCl, **$T_3$**: 100 mM $Na_2SO_4$, **$T_4$**: 150 mM NaCl + 100 mM $Na_2SO_4$

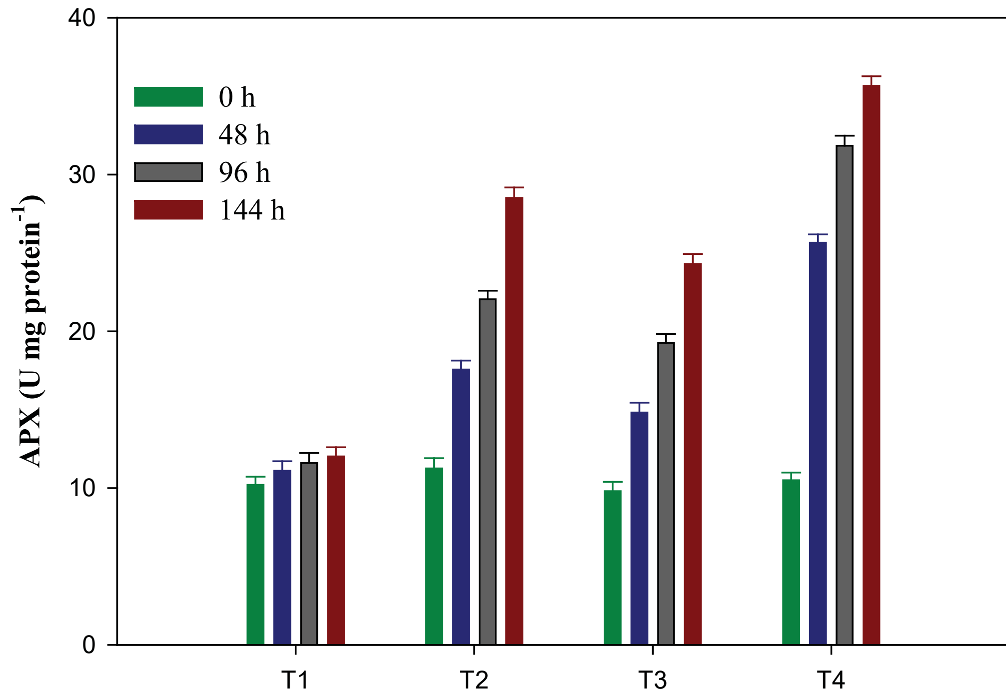



**Fig. 4.17. Effect of salinity on the ascorbate peroxidase (APX) activity of the cyanobacterium *A. doliolum*.**

**$T_1$**: Control, **$T_2$**: 150 mM NaCl, **$T_3$**: 100 mM $Na_2SO_4$, **$T_4$**: 150 mM NaCl + 100 mM $Na_2SO_4$

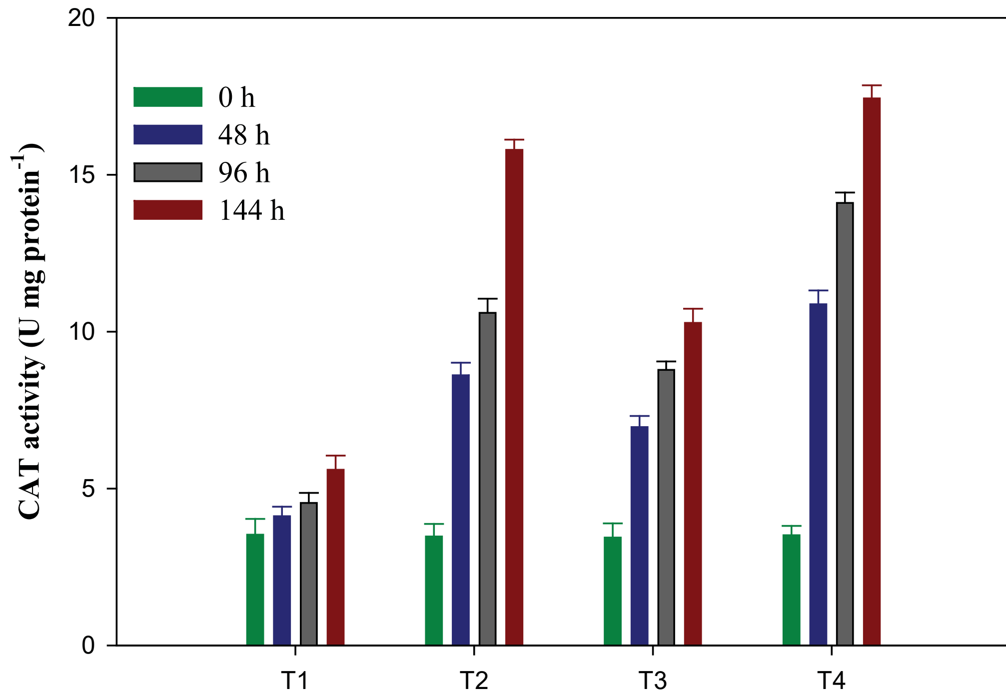



**Fig. 4.18. Effect of salinity on the catalase (CAT) activity of the cyanobacterium *A. doliolum*.**

**$T_1$**: Control, **$T_2$**: 150 mM NaCl, **$T_3$**: 100 mM $Na_2SO_4$, **$T_4$**: 150 mM NaCl + 100 mM $Na_2SO_4$

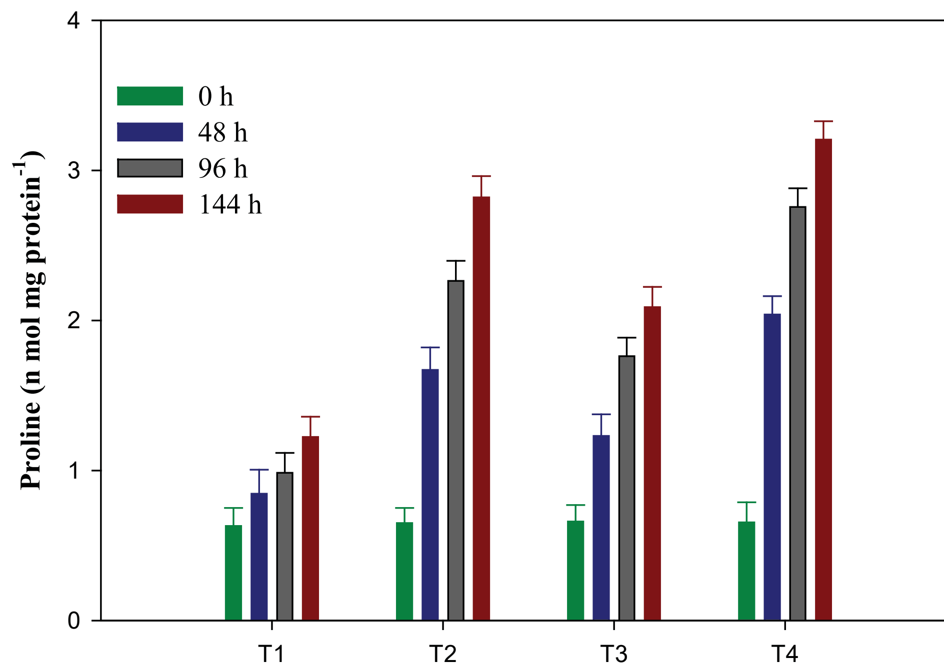



**Fig. 4.19. Effect of salinity on the proline accumulation by the cyanobacterium *A. doliolum*.**

**$T_1$**: Control, **$T_2$**: 150 mM NaCl, **$T_3$**: 100 mM $Na_2SO_4$, **$T_4$**: 150 mM NaCl + 100 mM $Na_2SO_4$

*Chapter 5*

# DISCUSSION

Due to the distribution of cyanobacteria in a number of ecological habitats they can be employed as model to study the response towards stressors (Friedmann and Galun, 1974). Murata and Wada (1995) observed that the cyanobacteria have survived exposure to a variety of stresses both man made and artificial and therefore will provide excellent clues for the environmental responses. Moore (1998) and Belnap (2003) observed that the cyanobacteria are pioneer colonizers in bare soils leading to the development of soil ecosystems. Therefore, Jones *et al.,* (1994) rightly mentioned them as "ecosystem engineers". Plant productivity and soil fertility are by affected due to salinity (Pitman and Lauchli 2002; Wong *et al.,* 2010; Amini *et al.,* 2016). Further, it is well established that the salinity also have a decisive role in determining the community structure of the microbes (Oren 2008: Yang *et al.,* 2016). Further, the level of salinity has also been reported to negatively affect the metabolism (Rietz and Haynes 2003; Chowdhury *et al.,* 2011). Therefore, understanding the tolerance mechanism in cyanobacteria to salt is important. Most of the studies conducted on cyanobacteria as well as other organisms are performed with NaCl. However, in the natural environment a mixture of different salts such as sodium, calcium and magnesium and anions such as chlorides, sulphates and bicarbonates are also present in toxic forms (Grattan and Grieve 1999). Moreover, in several parts of the country the agricultural soils are irrigated with saline water due to paucity of good quality irrigation water. $Na_2SO_4$ is prevalent in agricultural soils irrigated with saline water (Nriagu, 1978). There have been several reports on the effect of salinity induced perturbations on the growth of cyanobacteria (Srivastava *et al.,* 2008; Rai *et al.,* 2014; Swapnil *et al.,* 2015; Yadav *et al.,* 2016). However, the physiological basis of sulphate toxicity is poorly investigated. Hence it is important to assess as to how individual as well as a mixture of sulphate and chloride affect the growth of the cyanobacteria.

Therefore, understanding the tolerance mechanism to salinity stress induced by NaCl and $Na_2SO_4$ is important. It was observed in the present investigations that the growth of *Anabaena doliolum* decreased in response to individual as well as combined exposure to NaCl and $Na_2SO_4$ and the inhibition in growth was less pronounced in

response to $Na_2SO_4$. Growth estimated as increment in dry weight was declined due to NaCl and $Na_2SO_4$. Greater inhibition in the growth was observed due to combined exposure to both the salts. NaCl could lead to inhibition in the growth in response to salinity stress. Salinity stress induced reduction in the growth of cyanobacteria due to NaCl and $Na_2SO_4$ has been reported by Swapnil *et al.,* (2018). Apte, (2001) and Srivastava *et al.,* (2008) observed that loss of cellular water and turgidity induced by the influx of ions is one of the reasons for the decreased growth under salinity.

In the current investigation, the protein content of *A. doliolum* was affected by salinity. There are conflicting reports in response to the turn over of proteins in cyanobacteria in response to stress (Assche *et al.,* 1988; Jusu *et al.,* 2004). Lan *et al.,* (2010) reported that due to the synthesis of stress proteins, the protein content might generally exhibit an increasing trend. Salinity stress resulted in the degradation of several proteins in the cyanobacterium *Anabaena* (Ning *et al.,* 2002). Reduction in the protein profile of *A. doliolum* in response to salinity was reported by Srivastava *et al.,* (2008). Pade and Hagemann (2015) also reported the negative influence of NaCl on the protein content in cyanobacteria.

Salinity exposure exerted a negative impact on the pigments such as chlorophyll, carotenoid and phycobiliproteins such as phycocyanin, allophycocyanin and phycoerythrin. Boyer *et al.,* (2009) observed that chlorophyll content is considered as a measure of biomass. Stress in cyanobacteria resulted in inhibition of pigment synthesis (Lin and Wu 2014). Reduction in chlorophyll content in the cyanobacterium *Anabaena* sp under NaCl stress conditions was found (Sharma *et al.,* 2012). Reduction in the phycocyanin content due to salinity is in agreement with the findings of Lu and Vonshak (2002) and Marin *et al.,* (2004). Phycocyanin is highly sensitive to stress conditions because it is localized externally on intracellular thylakoid membrane (Grossman *et al.,* 1993; Prasad *et al.,* 2005). The main photosynthetic pigment chlorophyll along with the accessory pigments plays a lead role in photosynthesis. However, inhibition in the pigment content may result in decrease in growth due to inhibition in photosynthesis. Our results are supported by the findings of Neto *et al.,* (2014) and Yang *et al.,* (2014) who made similar observations under salinity stress. In general, the carotenoid content increases due to salinity stress conditions. Reports are available on the enhanced synthesis of carotenoids due to osmotic stress conditions (Lohscheider *et al.,* 2011; Lin and Wu, 2014). However, in the present study it was

observed that the carotenoid content decrease in response to individual as well as combined exposure to NaCl and $Na_2SO_4$. Ledford and Niyogi (2005) and Osmond *et al.,* (1997) highlighted the role of carotenoids in response to quenching of singlet $O_2$ and protecting cells against ROS. Therefore, it might be surmised that the carotenoids may not be required in the antioxidant machinery of the cells under stress conditions.

Increased accumulation of total soluble sugar content in response to salinity treatment was observed irrespective of the type of salinity. Reed *et al.,* (2002) observed that the accumulation of sugars such as trehalose and sucrose was observed in fresh water cyanobacteria with lowest level of salinity tolerance. Increase in the accumulation of sugars in many cyanobacteria has been reported earlier (Hershkovitz *et al.,* 1991; Higo *et al.,* 2006). Singh *et al.,* (2013) observed that the survival rate of *Anabaena* sp PCC 7120 under challenging conditions was enhanced due to accumulation of more sugars. Fisher (2006) observed that the accumulation of certain sugars especially trehalose prevented the *in vivo* aggregation of proteins.

The present study showed that NaCl salinity increased the intracellular $Na^+$ levels significantly in the cyanobacterium *A. doliolum* whereas the accumulation of intracellular $Na^+$ was low in response to $Na_2SO_4$ induced salinity. However, drastic increase the intracellular $Na^+$ content was observed due to salt mixture (NaCl + $Na_2SO_4$). Rejili *et al.,* (2007) observed ion toxicity, ion imbalance and nutrient deficiency due to high concentration of sodium ions. Intracellular $K^+$ content although increased in response to NaCl and $Na_2SO_4$, did not show much significant increase in relation to NaCl and $Na_2SO_4$. Potassium is important in the functioning of several biochemical processes (Pervez *et al.,* 2001; Romheld *et al.,* 2010). During salinity stress $Na^+$ content in the cells increase and replaces $K^+$ due to its structural similarity with $K^+$ leading to $K^+$ deficiency (Maathius and Amtmann, 1999). Salinity also resulted in higher cellular $Ca^{2+}$ content in a differential manner. Alterations in the intracellular $Ca^{2+}$ levels have been observed in response to salt stress (Kader and Lindberg (2010). Martinez *et al.,* (1993) and Kader *et al.,* (2008) observed that the channel activity was controlled by calcium by modulating the efflux of $Na^+$ and $K^+$ ions and provide stability to the membrane. Further, NaCl failed to maintain higher $K^+/Na^+$ ratio and the ratio reduced drastically in cells grown on salt mixture. Morant-Manceau *et al*., (2004) and Munns *et al.,* (2008) observed that the ratio of $K^+/ Na^+$ and $Ca^{2+}/ Na^+$ is important in relation to salinity tolerance. Enhanced intracellular sodium and calcium contents and

low $K^+$/ $Na^+$ ratio indicate growth inhibition due to loss of membrane permeability. These results are also supported by the findings of Swapnil *et al.,* (2018) who observed changes in the cyanobacterium *Anabaena fertilissima* due to NaCl and $Na_2SO_4$ salinity.

In the present study decrease in the average filament length and heterocyst frequency was observed due to salinity. In a clonal population, the average filament length and heterocyst frequency is constant but may vary with change in the composition of the growth medium. Decrease in average filament length and heterocyst frequency was seen in *Anabaena doliolum* due to salinity. Reduction in the heterocyst frequency may lead to reduced nitrogen fixation and this may lead to reduced protein synthesis (Ownby *et al.,* 1979). This can hamper the synthesis of proteins related to nitrogenase enzyme. The activity of nitrogen assimilation enzymes such as nitrate reductase, glutamine synthetase and nitrogenase was found to be inhibited significantly in response to salinity treatment. Nitrate is a major reservoir of nitrogen in the environment and its reduction to ammonia is essential in nitrogen assimilation. Swapnil *et al.,* (2015) correlated higher levels of the nitrate reductase activity with less toxicity due to stress conditions. However, in thecurrent investigation we have seen that the nitrate reductase activity is decreased due to salinity indicating toxic effects of salinity. The glutamine synthetase activity was also found to decrease in *A. doliolum* due to salinity exposure. Increase in the glutamine synthetase in *Anabaena* sp due to salinity stress was reported (Swapnil *et al.,* 2015).But Warr *et al.,* (1984) found that increase in the glutamine synthetase activity demands high energy. Therefore, the decreased glutamine synthetase activity could be due to the energy crisis and diversion of metabolic energy for survival (Apte *et al.,* 1987; Reddy *et al.,* 1989). It was seen that the acetylene reduction activity inhibited in relation to salinity exposure. Decrease in the nitrogenase activity in cyanobacteria due to salt stress conditions was observed (Moisander *et al.,* 2002). Reduction in the nitorgenase in response to salinity has also been reported by Srivastava *et al.,* (2008) and Sen *et al.,* 2017).

It was observed in the present study that subjecting the cyanobacterium *A. doliolum* to differential salinity treatment resulted in the enhanced acuumulation of lipid peroxidation products. The accumulation of the lipid peroxidation products was however, more pronounced in a mixture of NaCl and $Na_2SO_4$ than NaCl and $Na_2SO_4$ alone. Increase in the accumulation of lipid peroxidation products indicate oxidative stress conditions induced by salinity. Increased levels of lipid peroxidation in

cyanobacteria exposed to stress was observed (Rahman *et al.,* 2015; Sen *et al.,* 2017). Hyper activity of the antioxidant enzymes was observed in *A. doliolum* subjected to salinity. The breakdown of the superoxide anion to $H_2O_2$ is catalyzed by the enzyme superoxide dismutase. Ascorbate peroxidase enzyme is essential in the removal of $H_2O_2$ with the help of ascorbate as donor of electrons (Asada *et al.,* 1987). Scandalios (1993) observed that cellular damage is prevented due to efficient functioning of catalase and superoxide dismutase. In many algal forms including cyanobacteria stresses enhanced the activity of antioxidant enzymes (Mallick and Mohn, 2000; Collen and Davison 2001; Abd El-Baky *et al.,* 2004). The reactive oxygen species generated during the stress conditions lead to impediments in the growth and development (Choudhury *et al.,* 2013). Increase in the antioxidant enzyme activities may therefore be aimed to overcome the salinity induced stress conditions and to maintain optimal cellular ion homeostasis. Involvement of superoxide dismutase (Canini *et al.,* 2001), ascorbate peroxidase (Rozen *et al.,* 1992) and catalase (Chakravarty *et al.,* 2016) for proper removal and scavenging of free radicals is well known in cyanobacteria. Exposure of the cyanobacterium *A. doliolum* to salinity enhanced proline accumulation. Proline has a role in protecting the macromolecules and it also takes part in osmoregulation (Nikolopoulos and Manetas, 1991; Delauney and Verma 1993; Wu *et al.,* 1998). Increase in the proline content in cyanobacterial cells exposed to stress conditions has been observed (Sen *et al.,* 2017).

Negative impacts of salt on biomass production, carbon fixation and nitrogen metabolism in cyanobacteria have been investigated in detail (Srivastava *et al.,* 2005, 2008; Rai *et al.,* 2014; Sen *et al.,* 2015). However, there are very few reports available on the role of sulphate induced salinity in cyanobacteria. Paek *et al.,* (1988) observed that the toxicity induced by sulphate was severe as compared chloride. Detrimental effects of sulphate were also observed by Dixon (2007) in plants grown in environments receiving excess of sulphate in the soil. Sulphur is an important nutrient and its assimilation in to cysteine, which is a precursor of a variety of organic compounds (Hell *et al.,* 2002; Saito 2004; Hawkesford and De Kok 2006). Our results showed that salinity is detrimental to the growth of the nitrogen fixing cyanobacterium *A. doliolum*. However, the detrimental effects were more pronounced when the cyanobacterium was exposed to NaCl alone or in case of a mixture of NaCl and $Na_2SO_4$. Negative effect on the growth was exerted by both types of salinity. Rogers *et al.,* (1998) found that $Cl^-$

has a significant interfering effect as compared to $SO_4^{2-}$ in relation to plant growth and maintain the water balance. Ning *et al.,* (2015) observed that the oxidative stress conditions created by NaCl has far reaching consequences than $Na_2SO_4$. Lesser toxic effects observed in the cyanobacterium may be due to the presence of sulphur in the form of sulphate in the growth medium. Availability of sulphate in the growth medium probably have role in improving the antioxidant machinery status of the cells. Presence of sulphur in the nutireint medioum was found to enhance the cellular levels of the redox thiol compounds such as thiorexin, glutaredoxin and peroxiredoxin (Masip *et al.,* 2006; Foyer *et al.,* 2009; Cameron and Pakrasi 2010). Sulphate supplementation also has a role increasing the levels of GSH as observed by Schafer and Buettner (2001). Cells exposed to NaCl and $Na_2SO_4$ in combination exhibited better GSH levels as compared to NaCl alone. Cameron and Pakrasi (2010) observed decrease in the GSH levels due to sulphate deprivation. The physiological basis of sulphate induced toxicity in cyanobacteria is poorly understood. Reich *et al.,* (2017) observed that growth inhibition due to sulphate was more pronounced than chloride. Studies conducted by Swapnil and Rai (2018) also made similar observations in the cyanobacterium *Anabaena fertilissima* exposed to salinity NaCl and $Na_2SO_4$ either individually or in combination. Modulation of the $Na^+$ and $K^+$ ions in response to differential level of salinity was also in agreement with the findings of Swapnil and Rai (2018). It was conclusively shown by the present investigation that both the salinities resulted in significant reduction in the growth and the inhibition was less pronounced in case of sulphate induced salinity. The results show the salinity tolerance capacity of *Anabaena doliolum* to both types of salinity. Despite decrease in growth and nitrogen metabolism increase in the activity of antioxidant enzymes as observed in the current study may also not lead to salt tolerance. However, availability of sulphur to the plant may help the cyanobacterium in acquiring salinity tolerance. Hence, the sulphur nutrition may probably be used for better management of salinity stress in cyanobacteria and the efficient utilization of cyanobacteria in improving the nitrogen economy of the saline soils. Multipronged strategies are also required employing stress tolerant cyanobacterial strains to combat the ever increasing salinization of the arable land. For this the stress tolerance mechanisms need to be unraveled using promising strains of cyanobacteria having considerable levels of tolerance against chloride and sulphate induced salinity.

*Chapter 6*

# SUMMARY AND CONCLUSIONS

The cyanobacteria or blue green algae are eco-friendly and low cost inputs used in sustainable agriculture. They are important organisms in the nitrogen and carbon metabolism and are important as primary producers in any ecosystem. The cyanobacteria are used as food supplements for humans and therefore exploited industrially. However, commercial exploitation and application as bioinoculant is severely restricted due to the increase in salt levels in soils which is posing danger to the agriculture. Cyanobacterial communities can be found in several types of extreme environments. The salinity tolerance of agriculturally important microorganisms like cyanobacteria is not understood properly. Cyanobacteria develop several strategies at the cellular level to survive and grow under saline environment. Majority of the investigations on salt stress are conducted with sodium chloride (NaCl) but in the natural environment, a mixture of different salts such as sodium, calcium and magnesium and anions such as chlorides, sulphates and bicarbonates are also present. A number of reports are available on the impact of NaCl induced salinity on cyanobacteria. However, no attempts have ever been made to study the combined effect of chloride and sulphate induced salinity in cyanobacteria. Therefore, the present study entitled "Studies on the effect of chloride and sulphate induced salinity on the growth and physiology of the cyanobacterium *Anabaena doliolum*" was conducted. In agricultural soils irrigated with saline water $Na_2SO_4$ is prevalent and in the ecological niches, the organisms are exposed to selection pressure due to more than one stress factor.

The nitrogen fixing *Anabaena doliolum* was incubated in growth medium supplemented with NaCl and $Na_2SO_4$ (0-250 mM) individually for 8 days. Growth was estimated in terms of chlorophyll and it was observed that the cyanobacterium tolerated NaCl upto 150 mM whereas, it tolerated $Na_2SO_4$ upto 100 mM. NaCl (150 mM) and $Na_2SO_4$ (100 mM) salinity inhibited the growth and a mixture of NaCl (150 mM) and $Na_2SO_4$ (100 mM) resulted in further reduction of growth. The protein of the cyanobacterium also exhibited a declining trend similar to growth. Significant reduction in the pigment profile (Chlorophyll, carotenoid, phycocyanin, allophycocyanin and phycoerythrin) was also seen due to salinity treatment indicating

impairment in photosynthesis. While most of the cellular constituents decreased in response to salinity, the sugar content was found to increase in the cyanobacterium. The level of cellular sugar content enhanced with time and reached their maximum value at 144 hour of salt exposure. Increased accumulation of certain sugars may help the organism in osmoregulation. Intracellular $Na^+$ levels increased due to NaCl induced salinity whereas cells exposed to $Na_2SO_4$ accumulated less $Na^+$. Intracellular $K^+$ and $Ca^{2+}$ content also increased in response to NaCl and $Na_2SO_4$. Cells subjected to sodium chloride (NaCl) and a combination of sodium chloride (NaCl) and sodium sulphate ($Na_2SO_4$) failed to maintain a favorable $K^+/Na^+$ and $Ca^{2+}/Na^+$ ratio. On the contrary, the $K^+/Na^+$ and $Ca^{2+}/Na^+$ ratio in response to $Na_2SO_4$ salinity was found to be almost at par with the control. Elevated intracellular $Na^+$ and $Ca^{2+}$ levels and low $K^+/ Na^+$ ratio indicate growth inhibition due to loss of membrane permeability.

Salinity treatment also reduced the filament length and heterocyst frequency of the cyanobacterium *A. doliolum*. Decrease in average filament length and heterocyst frequency is also an indicator of reduced growth and nitrogen assimilation. Salinity exposure also resulted in the inhibition of nitrogen assimilation enzymes such as nitrate reductase, glutamine synthetase and nitrogenise. Exposure of *A. doliolum* to differential salt treatment resulted in the significant accumulation of lipid peroxidation products and it was more pronounced in a mixture of NaCl and $Na_2SO_4$ than NaCl and $Na_2SO_4$ alone. Increase in the accumulation of lipid peroxidation products indicate oxidative stress conditions due to salinity. NaCl and $Na_2SO_4$ resulted in up regulation of antioxidant enzymes such as superoxide dismutase, ascorbate peroxidise and catalase. Cells exposed to salinity also accumulated more proline. However, the reduction in growth, cellular constituents and the enzymes of nitrogen assimilation was more pronounced when the cyanobacterium *A. doliolum* was exposed to a mixture of NaCl and $Na_2SO_4$. The results indicated that the toxicity due to chloride is severe as compared to sulphate.

**STUDIES ON THE EFFECT OF CHLORIDE AND SULPHATE INDUCED SALINITY ON GROWTH AND PHYSIOLOGY OF THE CYANOBACTERIUM *ANABAENA DOLIOLUM***

## ABSTRACT

In the present investigation, the effect of chloride and sulphate induced salinity on the growth and physiology of the cyanobacterium *Anabaena doliolum* was studied. Both sulphate and chloride decreased growth and cellular constituents such as protein, chlorophyll and phycobiliproteins of the test cyanobacterium. The sugar content was found to increase in the cyanobacterium with time and attained their maximal limit at 144 hour of salt exposure. Intracellular $Na^+$ levels increased due to NaCl induced salinity whereas cells exposed to $Na_2SO_4$ accumulated less $Na^+$. Intracellular $K^+$ and $Ca^{2+}$ content also increased in response to NaCl and $Na_2SO_4$. Cells exposed to NaCl and a mixture of NaCl and $Na_2SO_4$ failed to maintain a favorable $K^+/Na^+$ and $Ca^{2+}/Na^+$ ratio. On the contrary, the $K^+/Na^+$ and $Ca^{2+}/Na^+$ ratio in response to $Na_2SO_4$ salinity was found to be almost at par with the control. Salinity treatment also resulted in decrease in the filament length and heterocyst frequency of the cyanobacterium *A. doliolum*. Inhibition in the activity of the nitrogen assimilation enzymes such as nitrate reductase, glutamine synthetase and nitrogenase was observed in response to salinity. Exposure of *A. doliolum* to differential salt treatment resulted in the significant accumulation of lipid peroxidation products. Increase in the activity of antioxidant enzymes and accumulation of proline was also observed in response to salinity induced by NaCl and $Na_2SO_4$. However, the reduction in growth, cellular constituents and the enzymes of nitrogen assimilation was more pronounced when the cyanobacterium *A. doliolum* was exposed to a mixture of NaCl and $Na_2SO_4$. The results showed that chloride was more toxic to the growth of the cyanobacterium *A. doliolum* as compared to sulphate. However, a combination of chloride and sulphate had a greater impact on the growth and physiological parameters of the cyanobacterium *A. doliolum*.

Keywords: Cyanobacteria, *Anabaena*, Salinity, Growth

## सायनोबैक्टेरियम एनाबीना डोलियोलम की वृद्धि एवं कार्यिकी पर क्लोराइड एवं सल्फेट प्रेरित लवणता के प्रभाव का अध्ययन


## सारांश

प्रस्तुत अध्ययन में *सायनोबैक्टीरियम एनाबीन डोलियोलम* की वृद्धि एवं कार्यिकी पर क्लोराइड एवं सल्फेट प्रेरित लवणता के प्रभाव का अध्ययन किया गया है। परीक्षण किए गए सायनोबैक्टीरियम की वृद्धि तथा कोशिकीय घटकों जैसे कि, प्रोटीन, हरितवर्ण एवं फायकोबिलीप्रोटीन में सल्फेट एवं क्लोराइड, दोनों कमी करते हैं। समय के साथ सायनोबैक्टीरियम के शर्करा अंश में बढोतरी पायी गई और इसने लवण के साथ रहने के 144 घंटे पर अधिक सीमा को प्राप्त किया। NaCl के कारण परेरित लवणता में अंतराकोशिकीय $Na^+$ स्तरों में वृद्धि हुई जबकि $Na_2So_4$ के साथ एक्सपोज कोशिकाओं ने कम $Na^+$ का संचयन किया। NaCl एवं $Na_2SO_4$ की अनुक्रिया में अंतराकोशिकीय $K^+$ एवं $Ca^{2+}$ अंश में भी बढ़ोतरी हुई। NaCl तथा NaCl एवं $Na_2SO_4$ के मिश्रण के साथ एक्पोज कोशिकाएं, एक अनुकूल $K^+/Na^+$ एवं $Ca^{2+}/Na^+$ अनुपात बनाऐ नहीं रख सकीं। इसके विपरीत, $Na_2So_4$ लवणता की अनुक्रिया में $K^+/Na^+$ एवं $Ca^{2+}/Na^+$ अनुपात, कंट्रोल के लगभग समकक्ष पाया गया। लवणता उपचार के परिणामस्वरूप सायनोबैक्टीरियम *ए. डोलियोलम* के तंतु की लम्बाई तथा हेटेरोसिस्ट की बारम्बारता में भी कमी हुई। लवणता की अनुक्रिया में नाइट्रोजन स्वांमीकरण एंजायम जैसे कि, नाइट्रेज रिडक्टेज, ग्लूटेमाइन सिंथेटेज एवं नाइट्रोजिवेज की सक्रियता का संदमन भी देखा गया। *ए. डोलियोलम* को परिवर्तनशील मृदा उपचार के साथ एक्सपोज करने के परिणामस्वरूप लिपिड परॉक्सीडिशन उत्पादों का महत्वपूर्ण रूप से संचयन हुआ। NaCl एवं $Na_2SO_4$ द्वारा प्रेरित लवणता की अनुक्रिया में प्रतिऑक्सीकारक एंजायम की सक्रियता में बढ़ोतरी तथा प्रोलीन का संचयन भी देखा गया। तथापि वृद्धि, कोशिकीय संघटकों एवं नाइट्रोजन का स्वांगीकरण करने वाले एंजायम में तब अधिक सुस्पष्ट कमी देखी गई जब सायनोबैक्टीरियम *ए. डोलियोलम* को NaCl एवं $Na_2So_4$ के मिश्रण के साथ एक्सपोज किया गया। परिणामों ने दर्शाया कि एल्फेट की तुलना में क्लोराइड, सायनोबैक्टीरियम *ए. डोलियोलम* की वृद्धि के लिए अधिक विषाक्त (टॉक्सिक) था। तथापि क्लोराइड एवं सल्फेट के एक संयोजन का, सायनोबैक्टीरियम *ए. डोलियोलम* के वृद्धि एवं कार्यिकीय प्राचलों पर एक योगवाही (सिनर्जिस्टिक) प्रभाव देखा गया।

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